Biochimica et Biophysica Acta 1798 (2010) 2190–2200

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

Delivering cargoes into cancer cells using DNA aptamers targeting internalized surface portals

Erik W. Orava [a,c], Nenad Cicmil [b], Jean Gariépy [a,b,c,*]

[a] Department of Pharmaceutical Sciences, University of Toronto, Toronto, Ontario, Canada M5S 3M2
[b] Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada M5G 2M9
[c] Sunnybrook Research Institute, 2075 Bayview Avenue, Toronto, Ontario, Canada M4N 3M5

ARTICLE INFO

Article history:
Received 25 November 2009
Accepted 3 February 2010
Available online 6 February 2010

Keywords:
Antibody
Anticancer agent
Aptamer
Chemotherapy
Drug delivery
Nanomedicine
SELEX

ABSTRACT

Many evolving treatments for cancer patients are based on the targeted delivery of therapeutic cargoes to and into cancer cells. The advent of monoclonal antibodies and the use of peptide hormones, growth factors and cytokines have historically provided a spectrum of ligands needed to selectively target tumor-associated antigens on cancer cells. However, issues linked to the size, cost and immunogenicity of protein-based ligands have led to the search for alternate ligand families. The advent of short synthetic oligonucleotide ligands known as aptamers now provides a simple strategy to select for membrane-impermeant aptamers tailored to precisely target internalized surface markers present on cancer cells. Here we described how 25-base long, synthetic single-stranded DNA aptamers were derived to bind to known internalized tumor markers such as CD33, CEA, MUC1 and Tn antigens and are imported through these surface portals into cancer cells. The key consequence of using internalized aptamers is their ability to accumulate inside the cells, thus routing their therapeutic cargoes to intracellular sites relevant to their action. Internalized aptamers are discussed in the context of how such ligands have been used to create a range of guided therapeutic agents ranging from drug-based conjugates up to targeted nanoparticles.

Contents

Abbreviations: LNA, locked nucleic acid; CTLA-4, cytotoxic T-cell antigen-4; VEGF, vascular endothelial growth factor; AMD, age-related macular degeneration; PEG, polyethylene glycol; PSMA, prostate-specific membrane antigen; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PLK1, polo-like kinase 1; BCL2, B-cell leukemia/lymphoma 2; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CEA, carcinoembryonic antigen; CEACAM, carcinoembryonic antigen cell adhesion molecule; MUC1, mucin 1; PTK7, protein tyrosine kinase 7; MAG_2, mercapto-acetyl diglycine; ^{99m}TC, Technetium-99m

* Corresponding author. Sunnybrook Research Institute, 2075 Bayview Avenue, Toronto, Ontario, Canada M4N 3M5. Tel.: +1 416 480 5704.
E-mail address: gariepy@sri.utoronto.ca (J. Gariépy).

0005-2736/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2010.02.004

1. Introduction

Many classes of oligonucleotides such as siRNAs, microRNAs and antisense oligonucleotides represent potential therapeutic agents in view of their ability to selectively block the expression or transcription of genes and mRNAs inside diseased cells. Unfortunately, their anionic character makes them cell-impermeant and thus will not reach their intracellular targets unless they are conjugated or complexed to a cell-penetrating peptide, a polymeric vector, a protein ligand (hormones, cytokines, and monoclonal antibodies), a nanoparticle or a liposome favoring their import into cells or are delivered using a viral vector. A more recent and potentially simpler solution to this challenge is to derive short synthetic oligonucleotides known as DNA and RNA aptamers which themselves specifically bind to internalized surface markers (cellular portals) and thus can act as delivery vehicles for therapeutic oligonucleotides and other therapeutic cargoes. This review will provide a basic description of the principles underlying the concept and discovery of aptamers with a particular emphasis on targeting known internalized tumor cell surface markers.

1.1. The SELEX procedure: a rapid strategy to identify short single-strand synthetic oligonucleotides (aptamers) that recognize specific targets

Cancer cells typically harbor multiple oncogenic mutations leading to the aberrant display and/or overexpression of molecular signatures on their surface. Classical approaches to target such signatures have made use of peptides, proteins and mainly antibodies. However, recent studies suggest that oligonucleotides known as aptamers can be utilized in the same capacity. Aptamers are short single-stranded nucleic acid oligomers (ssDNA or RNA) that can form specific and complex three-dimensional structures which can bind with high affinity to specific targets. The term 'aptamer' is derived from the Latin word *aptus* meaning "to fit" [1]. Two groups reported a PCR-based strategy termed SELEX (an acronym for *S*ystematic *E*volution of *L*igands by *EX*ponential enrichment; [2]) to derive aptamers that specifically recognized targets ranging from small molecules to large proteins (Refs. [1,2]; Fig. 1). SELEX is an iterative panning procedure where combinatorial libraries composed of a random oligonucleotide element flanked by constant primer regions are allowed to bind to an immobilized target. The bound oligonucleotides are then recovered

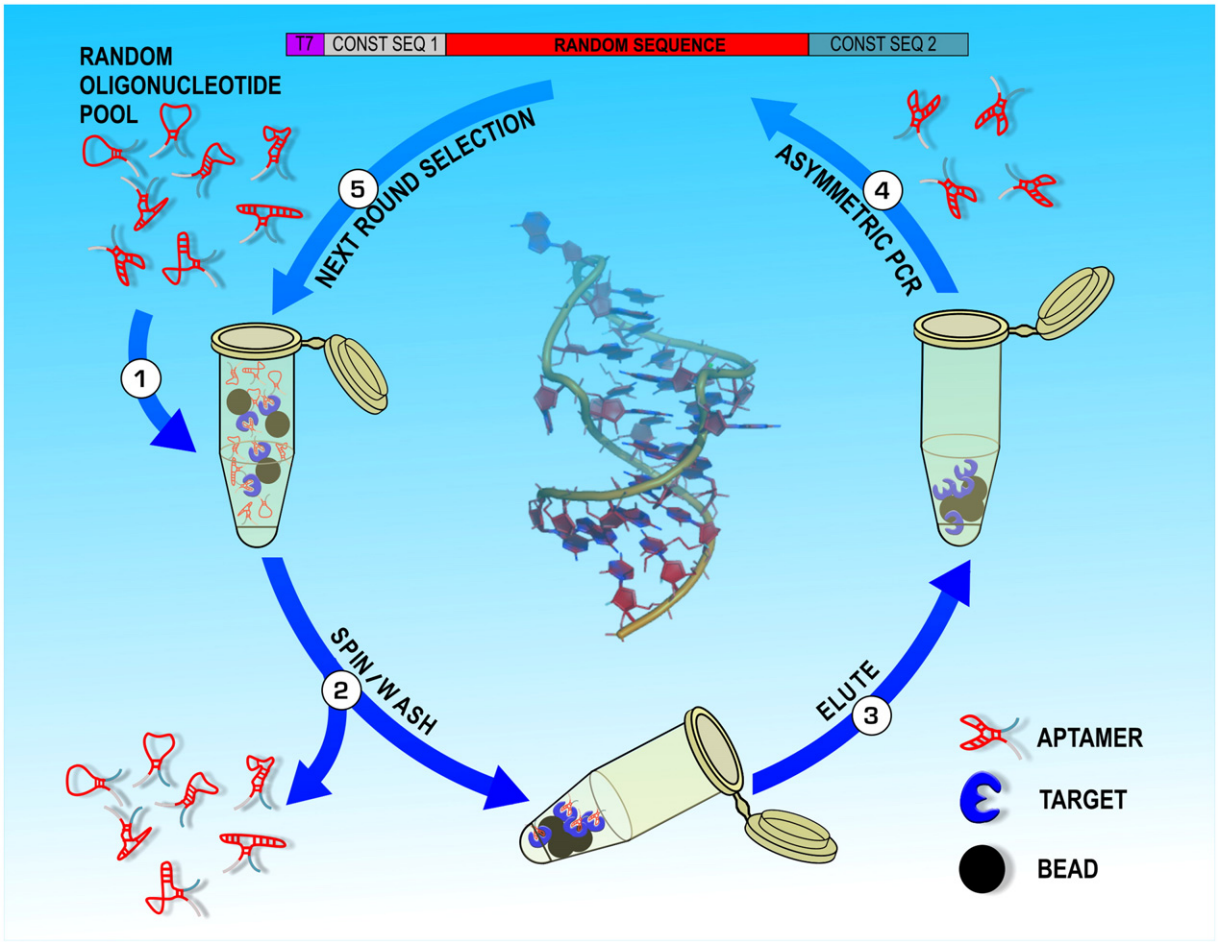

Fig. 1. Isolation of aptamers using the SELEX procedure. A combinatorial library of DNA oligonucleotides is chemically synthesized using standard solid-phase methods. The library is composed of a random oligonucleotide element sequence (typically 20 to 50 nucleotides in length) flanked by two distinct constant sequences for the subsequent enrichment of target-binding library elements by PCR. The synthetic DNA oligonucleotide pool is directly mixed with the immobilized target for the purpose of retaining bound DNA aptamers (step 1). In the case of an RNA aptamer selection, a random library of RNA aptamers is initially derived from a double-stranded DNA library by *in vitro* transcription, in which case the 5′ constant sequence includes a T7 promoter region. Aptamers in each library will adopt different three-dimensional structures based on their random sequence element with some oligonucleotides able to bind to a target immobilized on beads or other solid supports (step 1). The crystal structure of a thrombin-binding RNA aptamer [4] is displayed (center) to emphasize the presence of bulges and hairpins in such structures. Following a washing step (step 2), the bound oligonucleotides are eluted from the solid support (step 3) and amplified by PCR using the constant flanking sequences acting as primer sites (step 4). The selection cycle is repeated (step 5) typically 10–15 times with increasing stringency (lower target concentration for example) until tight-binding aptamers for a given target are identified, sequenced and synthesized for subsequent analyses.

and amplified by PCR to generate a sub-library of aptamers able to recognize a given target. The binding/amplification cycle is then repeated several times on enriched pools of aptamers until one recovers ssDNA or RNA aptamers displaying K_ds in the nanomolar to picomolar range for their respective targets. So far, thrombin represents the only protein that does not normally bind nucleic acids and for which crystals structures of its complexes with aptamers have been obtained [3,4]. Interestingly, the two available structures (thrombin complexed to a DNA and a RNA aptamer) indicate that each aptamer binds to a distinct region on the protein located on opposite sides of each other on the molecule (Fig. 2). This finding suggests that the process of identifying aptamers using the SELEX procedure does not necessarily favor a unique epitope on a given target. Specifically, the DNA aptamer was shown to contact a region of thrombin that normally binds to fibrinogen (exosite 1), while the RNA aptamer binds to a domain associated with heparin-binding (exosite 2) [5]. Interactions between these aptamers and thrombin are mostly electrostatic since both of the exosites are positively charged interfaces [3,5,6]. These structural features highlight the fact that aptamers recognize their targets mostly through electrostatic interactions in contrast to dominant hydrophobic interactions typically observed in proteins. It also suggests that the number of surface elements on a given target that could serve as recognized interfaces for aptamers is finite and potentially predictable.

1.2. RNA versus DNA aptamers

A large number of RNA aptamers have now been reported against different targets. The versatility of RNA molecules as functional ligands is well documented in regards to the frequent occurrence of modified nucleotides within their structure, their base pairing properties and their tendency to form intricate three-dimensional structures [7]. For instance, all natural riboswitches (which bind to small molecules) are RNA molecules [8]. The derivation and use of RNA aptamers does present some important practical challenges. For instance, the SELEX process requires the synthesis of random oligonucleotide libraries and the chemical synthesis of random RNA oligonucleotide pools remains expensive. Therefore, an *in vitro* transcription step is introduced in the SELEX procedure to obtain the initial RNA pool. Secondly, RNA oligonucleotides are more susceptible to hydrolysis than their DNA counterparts and thus their manipulation requires RNAse-free conditions.

DNA tertiary structures have been observed in nature [9]. These structures, rich in guanine, are found in telomeres and promoter regions [10,11]. Guanine-rich sequences form various G-quadruplexes that appear to be major structural elements found in DNA aptamers as exemplified in the thrombin DNA aptamer (Fig. 2). Examples of DNA aptamers have been reported and include an anti-HIV aptamer [12] and the anti-nucleolin aptamer AS1411 [13]. Catalytically-active DNA aptamers have also been derived using the SELEX approach [14,15]. The selection procedure for DNA aptamers is simpler than for RNA aptamers. Specifically, inexpensive pools (libraries) of DNA oligonucleotides can be chemically synthesized and contain only single-stranded sequences as opposed to the initial double-stranded pool of DNA sequences required for the *in vitro* transcription step used for RNA-based aptamer selection. Furthermore, reverse transcription is not required and an asymmetric PCR step is sufficient to recover the sub-library of ligand-binding aptamers needed to proceed to the next round of selection. In summary, the advantages of DNA aptamers stem from the simpler enrichment procedure involved and the lower cost and stability of the final aptamers while the benefit of selecting for RNA aptamers is the higher level of structural diversity possible with RNA templates.

2. Aptamers can serve as intracellular delivery vehicles via their binding to known cancer-associated surface antigens

The main purpose of this review is to highlight the potential of membrane-impermeant oligonucleotides to serve as intracellular delivery agents if they can be engineered to target internalized surface markers on cancer cells. The best described surface determinant used for this purpose (Table 1) has been the prostate-specific membrane antigen (PSMA), a membrane protein overexpressed on the surface of prostate cancer cells. PSMA is internalized by such cells via clathrin-coated pits [16–19]. From a drug delivery perspective, antibody studies have shown that the rate of PSMA internalization was promoted by the binding of an antibody to its extracellular domain [19]. The PSMA antigen is also differentially expressed on prostate cancer cells with normal prostate cells displaying an alternatively spliced cytosolic form of the protein while malignant cells express the full length surface protein [20]. The extracellular domain of PSMA served as a target for developing the first RNA aptamers known to bind a tumor-associated

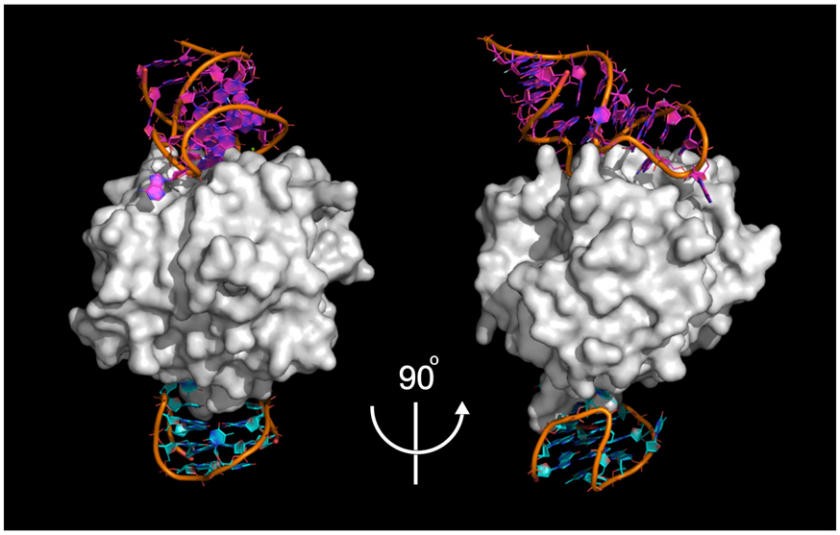

Fig. 2. Overlapped crystal structures of a RNA and a DNA aptamer bound to the protein thrombin [3,4]. The SELEX process has identified aptamers that interact with two distinct sites on thrombin (grey-colored contour surface). The 25-nucleotide long RNA aptamer Toggle-25t [purple, top] was found to bind near exosite 2 (heparin-binding site on thrombin) while the 15-base long DNA aptamer [blue, bottom] bound near the thrombin exosite 1 (fibrinogen-binding domain). Both interacting interfaces on thrombin displayed positively charged surfaces.

Table 1
Examples of aptamers and their cargoes directed at internalized surface markers on cancer cells.

Reference	Aptamer	Internalized target	Cargoes
[109]	sgc8c	PTK7	Viral capsid
			Drugs
[64]	A10	PSMA	Doxorubicin
[63]	sgc8c	PTK7	Doxorubicin
[62]	5TR1	MUC1 tandem repeat	Chlorin e_6
[62]	5TRG2	Tn antigen	Chlorin e_6
[62]	GalNAc3	N-acetylgalactosamine	Chlorin e_6
			Toxins
[67]	A9	PSMA	Gelonin
			Nanostructures
[70]	sgc8c	PTK7	Nanorod
[72–75,110]	A10	PSMA	Nanoparticle
[111]	AS1411	Nucleolin	Nanoparticle
[112]	A9	PSMA	Quantum dot
[76]	A10	PSMA	Quantum dot–doxorubicin
			Radioisotopes
[68]	TTA1	Tenascin-C	^{99m}TC

antigen [18]. The selective delivery and uptake properties of such aptamers by prostate cancer cells led to the subsequent design of an RNA chimera incorporating a PSMA-specific aptamer (delivery vehicle) and a therapeutic siRNA that targets Polo-like kinase 1 (PLK1) and BCL2. This RNA aptamer-siRNA construct was shown to cause tumor regression in a xenograft model of prostate cancer [21]. These findings suggested that by choosing appropriate internalized surface markers on cancer cells, one may be able to develop aptamers that can serve as both cell targeting agents and intracellular delivery vehicles. We will now focus our discussion on recent evidence from our laboratory suggesting that DNA aptamers can indeed be generated against membrane-bound tumor markers that are recycled inside cells.

2.1. CD33

The CD33 antigen is a 67-kDa type 1 transmembrane glycoprotein that belongs to the superfamily of sialic acid-binding immunoglobulin-related lectins (siglecs; siglec-3) [22]. CD33 is expressed on early multilineage hematopoietic progenitors, myelomonocytic precursors, as well as more mature myeloid cells, monocytes, macrophages and dendritic cells [23–25]. Most adult and pediatric acute myeloid leukemia (AML) cases as well as 15–25% of acute lymphoblastic leukemia (ALL) cases are CD33-positive [26–29]. The presence of CD33 on AML blasts has led to the development of monoclonal antibody treatments that have been approved for AML patients that have relapsed. One of these anti-CD33 antibodies was conjugated to calicheamicin, a potent cytotoxic antibiotic that cleaves double-stranded DNA at unique sites. The resulting antibody–drug conjugate is commonly known as Gemtuzumab ozogamicin or Mylotarg (Wyeth Laboratories, PA, USA) [30,31]. Antibody-bound CD33 has been shown to be rapidly internalized by myeloid cells, a process that is largely modulated by its cytoplasmic immunoreceptor tyrosine-based inhibitory motifs [32,33]. A 26% response rate has been observed for AML patients treated in first relapse with Gemtuzumab ozogamicin as a monotherapy with a median disease-free-survival of 6·4 months in patients [34]. Surprisingly, there is no major loss of surface CD33 expression on leukemic blasts at relapse after Gemtuzumab treatment suggesting that alternate therapies targeting CD33-positive cell populations would be feasible and safe [35,36]. This finding would suggest the development and use of smaller and less immunogenic CD33-specific aptamers carrying less toxic cargoes than calicheamicin (hepatotoxicity) into CD33$^+$ cells. As a proof-of-concept, our group has recently developed 25-base long synthetic DNA aptamers against a recombinant form of CD33 to examine their ability to be internalized by myeloid (CD33$^+$) cell lines. As shown by flow cytometry and confocal microscopy (Fig. 3), one such CD33-specific Cy5-labeled DNA aptamer binds to (4 °C) and is internalized (37 °C) by CD33$^+$ cells within 90 min of exposing cells to this oligonucleotide. In contrast, no binding or cellular uptake was observed for a control aptamer (25-base long repeat of the sequence ATGC) identically modified with a Cy5 probe exposed to the same set of cell lines. Finally, neither aptamers bound to the CD33$^-$ cell line LP1. The dissociation constant (K_d) of this monomeric CD33-specific aptamer was calculated to be 17.3 nM suggesting that it is only ~10 fold less avid for its target than modified forms of the established bivalent-binding CD33-specific monoclonal antibody HuM195 [37]. These results suggest that DNA aptamers evolved to bind to the antigen CD33 can mimic the properties of anti-CD33 antibodies in terms of binding and being imported into CD33-positive cells.

2.2. Carcinoembryonic antigen (CEA)

The human carcinoembryonic antigen (CEA) is a 180 kDa GPI-linked cell glycoprotein and a member of an immunoglobulin cell adhesion molecule superfamily (CEACAMs). CEA was originally identified as a surface marker on adenocarcinomas of the human gastrointestinal tract as well as on cells of the fetal digestive system [38]. Other CEACAM members have since been identified in an array of tumors including breast, lung, pancreas, stomach, thyroid, ovaries and melanomas [39]. CEA is aberrantly overexpressed on the surface of colorectal tumor cells in relation to normal colonic cells [40]. As the tumor progresses and invades the basal lamina, elevated levels of CEA can be detected in sera. For this reason, CEA has been used as a serum marker for recurrence of colorectal cancer despite its low sensitivity and specificity [41]. CEA has often being referred to as a non-internalizing or as a shed antigen, yet studies have shown that anti-CEA antibodies are endocytosed at a rate consistent with the metabolic turnover of CEA [42–45]. Anti-CEA antibody targeted therapies have been reported to date [46,47]. As in the case of antibody therapies aimed at solid tumors, poor tumor penetration remains an issue and in the specific cases of high affinity CEA antibodies, their rapid clearance due by free circulating antigen [48,49]. In order to assess the potential of CEA as an internalizing antigen on cancer cells, DNA aptamers were developed specifically to recognize a recombinant form of the N-terminal Ig domain of human CEA using the SELEX approach. The binding of one such 25-base long DNA aptamer (and a control DNA aptamer) to the mouse colon adenocarcinoma cell line MC-38 (CEA$^-$) and its related cell line transduced to express the human CEA gene, MC-38.cea (CEA$^+$) [50] was monitored by flow cytometry. Specifically, these cells were incubated with a Cy5 conjugated CEA-specific DNA aptamer at 4 °C (surface binding only) and at 37 °C (binding and internalization). As shown in Fig. 4, MC-38 (CEA$^-$) MC38 cells showed no significant binding of the CEA-specific aptamer at both temperatures (Fig. 4B). In contrast, the CEA-specific aptamer strongly associated with the CEA-positive cell line MC-38.cea, with a significant increase in mean fluorescence intensity being observed after 2 h at 37 °C in relation to 4 °C (Fig. 4B). The higher fluorescence signal observed at 37 °C is attributed to the CEA aptamer being internalized during this time period. The irrelevant Cy5-labeled DNA aptamer (control; ATGC repeats) did not bind to either cell lines at both temperatures. Thus, CEA may represent a powerful portal for aptamer-directed conjugates to selectively reach and be imported into colon cancer cells.

2.3. CA15-3 antigen, MUC1 peptides and Tn antigens

The mucin MUC1 is a membrane glycoprotein that is highly expressed and is aberrantly glycosylated [shortened *O*-glycans structures] in greater than 90% of all primary and metastatic breast cancers [51–54]. The mucin MUC1 extracellular domain largely consists of 30

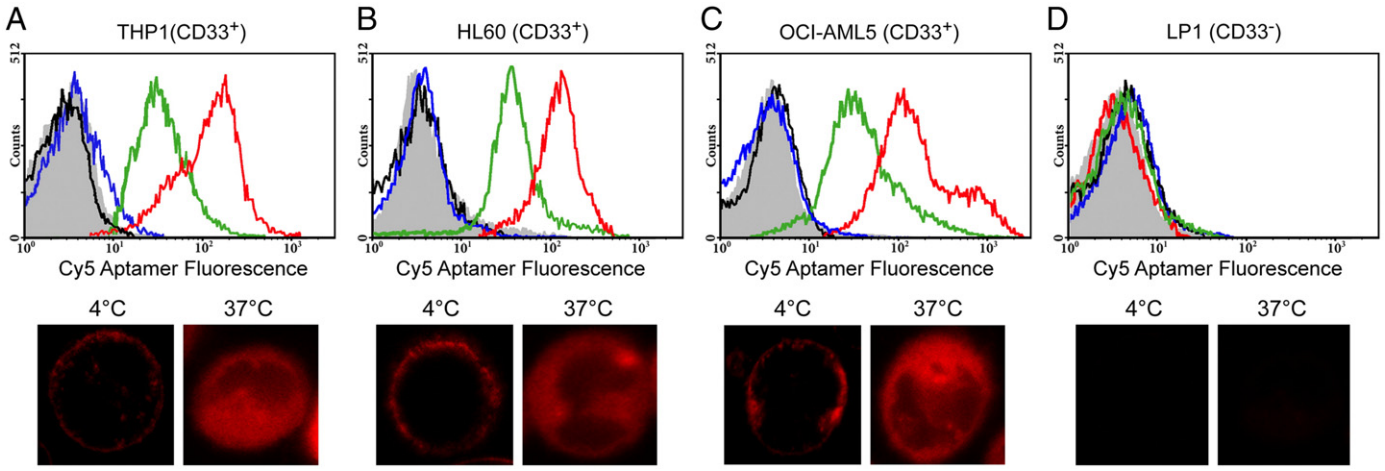

Fig. 3. A CD33-specific DNA aptamer binds to and is internalized by CD33$^+$ myeloid leukemia cell lines. FACS histograms (top) and fluorescence confocal images (bottom) of CD33$^+$ myeloid leukemia cell lines HL60, OCI-AML5 and THP1 exposed to a 25-base long synthetic, Cy5-labeled CD33-specific DNA aptamer at 4 °C and 37 °C for 90 min. The flow cytometry profiles indicate that the CD33-specific DNA aptamer binds specifically to CD33$^+$ cells at 4 °C (green curves) with increased fluorescence intensities being observed at 37 °C (red curves) as a consequence of internalization. In contrast, the labeled aptamer did not recognize the CD33$^-$ myeloma cell line LP1. A 25-base long Cy5-labeled control DNA aptamer composed of ATGC repeats displayed fluorescence profiles at 4 °C (black curves) and 37 °C (blue curves) that were comparable to the autofluorescence profiles (grey areas) of unstained cells.

to 100 copies of a 20-amino acid long tandem repeat [55]. Serine and threonine residues within the tandem repeat represent sites of *O*-glycosylation. The pattern of *O*-glycosylation at such sites is altered in cancer cells giving rise to truncated short sugar chains known as the T, Tn and sialyl-Tn antigens [56,57] as well as exposing antigenic sites on the peptide chain itself. MUC1 peptide domains and its associated truncated carbohydrate epitopes are clinically referred to as the CA15-3 antigen. Increasing serum levels of the CA15-3 antigen correlate with poor prognosis. In terms of drug delivery, mucin MUC1 glycoforms are endocytosed and recycled by cells in order to complete their glycosylation pattern prior to returning to the cell surface [58–61]. Any ligands binding to such structures will thus be imported into MUC1$^+$ cells and in particular through Golgi compartments. Our group has recently derived short 25-base long, synthetic DNA aptamers that specifically recognize either the MUC1 peptide backbone or its Tn antigens (GalNAc sugars linked to serine and threonine hydroxyl

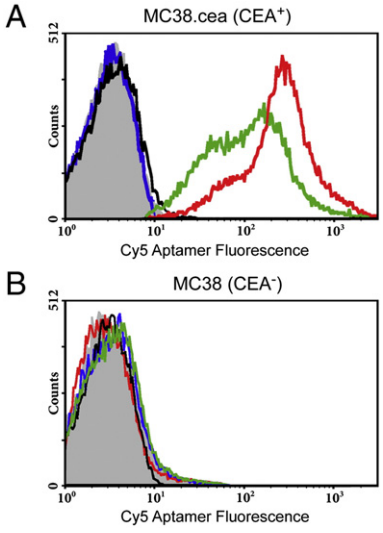

Fig. 4. A CEA-specific DNA aptamer binds to and is imported into colon carcinoma cells expressing human CEA. FACS histograms confirmed the binding (4 °C, green curves) and internalization (37 °C, red curves) of a 25-base long synthetic, Cy5-labeled CEA-specific DNA aptamer binding to the human CEA$^+$ MC38.cea cell line after a 2-hour incubation period. No binding or internalization was observed for the Cy5 aptamer to the parent CEA$^-$ MC38 cell line or for a control aptamer (as described in Fig. 3; black (4 °C) and blue (37 °C) curves) to both cell lines.

side chains on the MUC1 peptide tandem repeat) on epithelial cancer cells with binding affinities (K_ds) for their targets ranging from 18 to 85 nM [62]. Confocal microscopy and flow cytometry studies [62] have shown that these labeled aptamers circulate from the cell surface and into endosomal and Golgi compartments upon binding to underglycosylated mucins (Fig. 5). These DNA aptamers were subsequently derivatized at their 5′ end with the photodynamic therapy agent chlorin e_6 and shown to deliver chlorin e_6 to cellular compartments and cause cytotoxicity at concentrations 2- to 3-orders of magnitude lower than the concentration needed for the free drug [62].

3. Aptamer-guided delivery of payloads into cancer cells

In theory, aptamers represent simpler antibody-like mimics in terms of their ability to recognize tumor markers. Therapeutic agents can be directly coupled to aptamers or packaged into particles modified with aptamers in order to exploit recycling pathways associated with internalized cancer markers. However, the optimal efficacy of an aptamer-based intracellular delivery agent will depend in part on the recycling properties of their target and the possible induction of a receptor-mediated internalization event upon binding to a surface marker. In addition, the intracellular routing of aptamers is influenced by the abundance of the cell surface target itself, the macroscopic nature of the aptamer conjugate being delivered (size and nature of the cargo) and the dominant endocytic pathways associated with a given tumor cell type. The known cellular import mechanisms that lead to the vesicular trafficking of ligands bound to cell surface receptors are illustrated in Fig. 6 and include (1) macropinocytosis and (2) phagocytosis, distinguished by the size of their endocytic vesicles, (3) clathrin-mediated, (4) caveolae (caveolin-based lipid rafts) and (5) clathrin-independent pathways. Recently designed aptamer-cargoes complexes do exploit import pathways, although few studies have explored their mode of cellular delivery. Most reported examples of internalized aptamer conjugates (Table 1) have either made use of the RNA aptamers A9 and A10 directed at the prostate-specific membrane antigen (PSMA) or the DNA aptamer sgc8c recognizing the tyrosine kinase 7 (PTK7).

3.1. Aptamer–drug conjugates

Aptamer–drug conjugates have been constructed by chemically coupling a chemotherapeutic drug to the aptamer via a linker [63] or

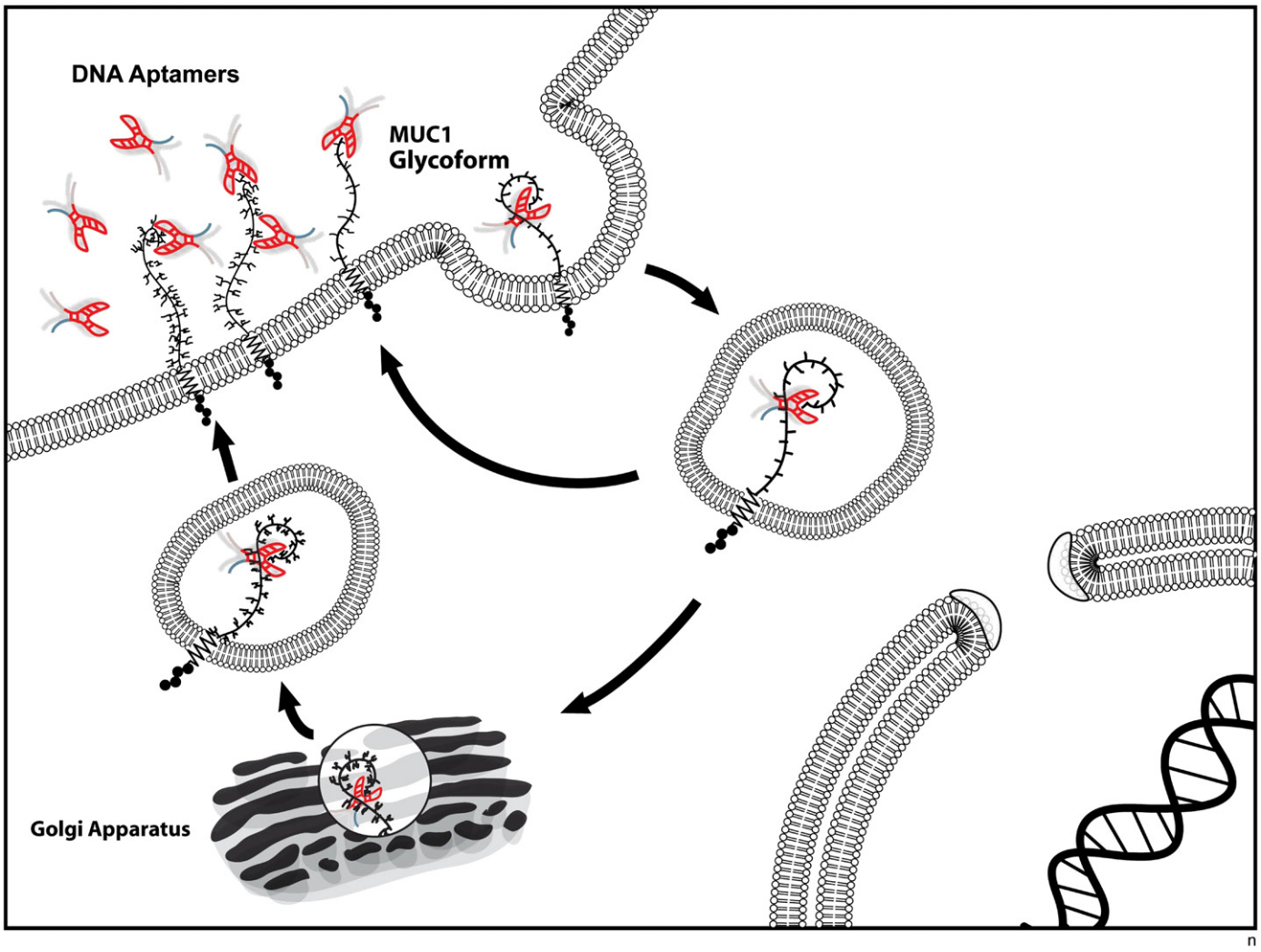

Fig. 5. Proposed mechanisms of cellular entry and recycling of DNA aptamers directed at aberrantly glycosylated mucin MUC1 present on the surface of epithelial cancer cells. Aptamers bind to membrane-bound, underglycosylated MUC1 mucin (branched structures). These mucin structures are recycled from the cell surface into endosomes that are either sent back to the cell surface or routed to the Golgi network where they are further glycosylated before returning to the cell surface.

by intercalating the drug into the aptamer folded structure creating a physical complex [64]. The drug is then imported into target cells while reducing its toxicity towards other cells (oligonucleotides including aptamers are cell-impermeant). Drugs can be conjugated to aptamers during solid-phase synthesis or post-synthesis by incorporating an amino or thiol group at one end of the oligonucleotide during their assembly. For instance, doxorubicin, an anthracycline used in the treatment of various cancers, has been coupled via an acid-labile hydrazone linker [65] to a 41-nucleotide long tyrosine kinase 7 PTK7-specific DNA aptamer (sgc8c) to release the drug in endosomes. This aptamer–drug conjugates has been shown to prevent the non-specific internalization of the drug as well as decrease its cellular toxicity towards non-target cells. The conjugate is selectively internalized by CCFR-CEM cells (T-cell acute lymphoblastic leukemia cells) with no apparent reduction in aptamer affinity for its target [63]. As mentioned in Section 2.3, DNA aptamers targeting known tumor-associated antigens such as mucin MUC1 peptides and mucin Tn antigens have also been modified with a photodynamic therapy agent chlorin e_6 and delivered to epithelial cancer cells. These aptamer–chlorin e_6 conjugates exhibited a >500-fold increase in toxicity upon light activation as compared to the drug alone and were not cytotoxic to cells lacking these mucin markers [62].

3.2. Aptamer–protein conjugates

Previous work with antibody–toxin conjugates has suggested that the most important determinant of cellular cytotoxicity of immunotoxins is the efficiency of their import into cells [66]. The coupling of aptamers to cytotoxic as well as therapeutic proteins can facilitate them reaching their intracellular substrates. A case in point is the anti-PSMA RNA aptamer (A9) conjugated to gelonin, a ribosome-inactivating protein toxin. As mentioned in Section 2, the prostate-specific membrane antigen (PSMA) is internalized by prostate cancer cells and thus provides a portal for the directed entry of the cytotoxic PSMA-specific aptamer-gelonin construct into such cells. Gelonin is an enzyme that inactivates ribosomes when deposited in the cytosol of intoxicated cells. The construct displayed a 600-fold increase in toxicity towards $PSMA^+$ LNCaP cells as compared to non-PSMA-expressing PC3 cells and ~180-fold increase in toxicity towards LNCaP cells relative to free gelonin [67].

3.3. Aptamer–radionuclide conjugates

Few aptamers to date have been modified to incorporate radionuclides or metal chelators with a view to image or kill cancer cells

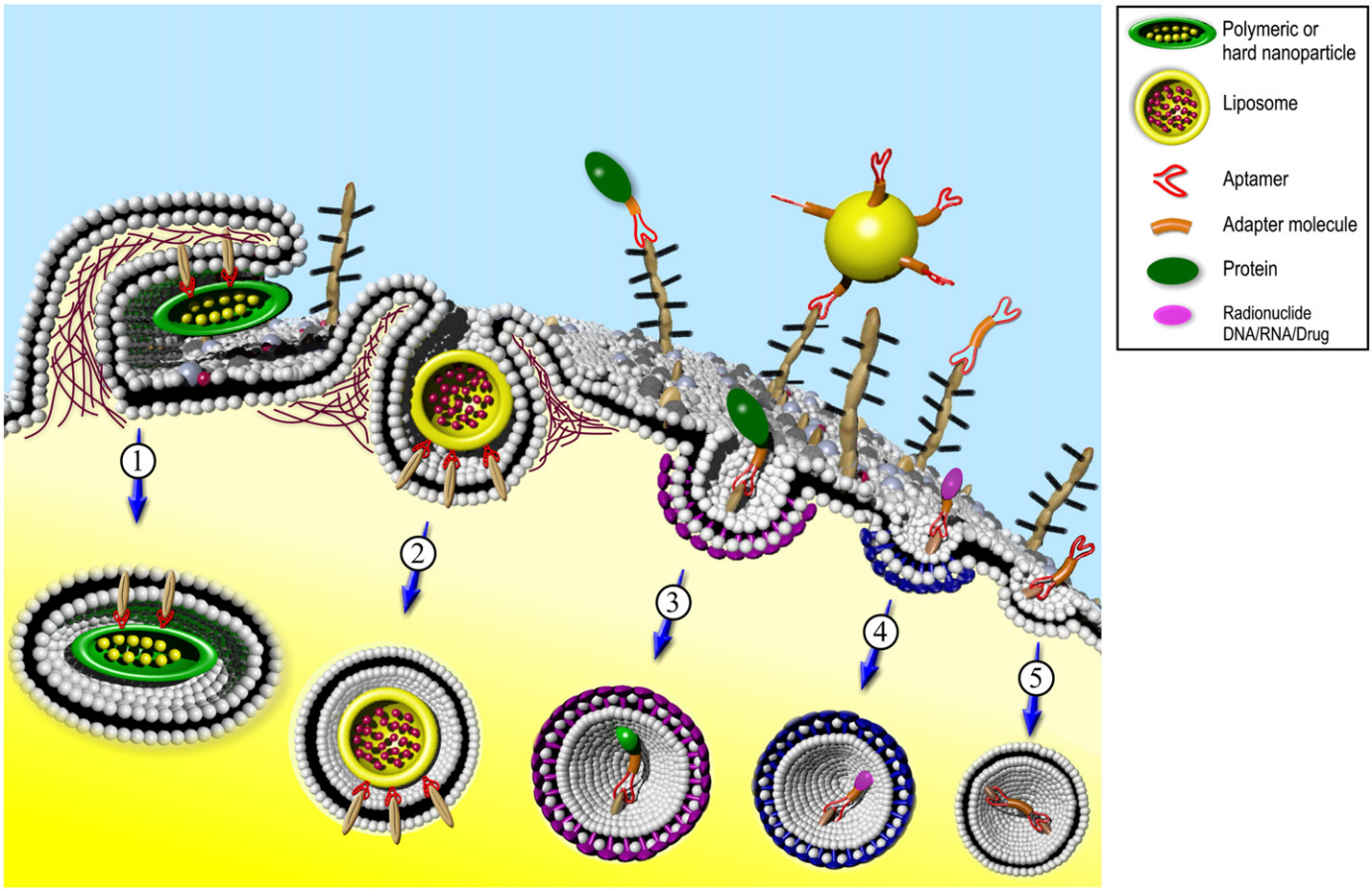

Fig. 6. Possible endocytic pathways taken by aptamer-cargoes. The nature and size of a cargo, the membrane-cycling property and abundance of a targeted surface portal on a given cell type as well as the number of portal-directed aptamers attached to a cargo (valency) can influence which import mechanism(s) may dominate in routing an aptamer-containing conjugate into cells. Large multivalent aptamer-targeted nanoparticles, polymer aggregates and liposomes would favor actin-filament-mediated uptake mechanisms such as macropinocytosis (1) or phagocytosis (2) while smaller monomeric aptamer conjugates involving a drug, a radionuclide, an siRNA or a protein as examples of payloads may enter cells via receptor-mediated events involving clathrin-dependent (3), caveolin-dependent (4) and/or clathrin-independent (5) endocytic pathways.

in vivo. Hicke et al. [68] have reported the introduction of the metal chelator mercapto-acetyl diglycine (MAG_2) at the 5′ end of TTA1, a Tenascin-C-specific aptamer. TTA1 is a 40-nucleotide long RNA aptamer that incorporates 2-fluoro-pyrimidines and binds to the protein Tenascin-C with a K_d of 5 nM [69]. Tenascin is a large, hexameric glycoprotein associated with the extracellular matrix and is expressed during tissue remodeling events linked to angiogenesis and tumor growth. The MAG_2-containing TTA1 aptamer chelates ^{99m}Tc and was used to determine its biodistribution *in vivo* in the context of nude mice harboring a human glioblastoma U251 xenograft. ^{99m}Tc-TTA1 showed rapid blood clearance and tumor uptake, reaching a tumor-to-blood ratio of 50 within 3 h. In addition, good scintigraphy images of a breast and glioblastoma tumor xenograft in nude mice were recorded using this labeled aptamer [68]. The success of this particular chelator–aptamer complex also highlighted the empirical nature of the design process as an alternate choice of a chelator and radionuclide does result in significant changes in the uptake and clearance patterns of this aptamer *in vivo*. Nevertheless, the use of radiolabeled aptamers for imaging purposes *in vivo* is feasible.

3.4. Aptamer–nanostructure conjugates

The recent creation of aptamer-conjugated nanostructures suggests that they may represent a promising class of new agents for targeted cancer imaging and therapy. These targeted structures include nanorods, quantum dots, as well as soft and hard nanoparticles. Nanorods for example, can be viewed as an alternate scaffold for assembling and immobilizing aptamers to nanomaterials in order to generate multivalent conjugates. Huang and colleagues were able to show that up to 80 aptamers could be covalently linked to the surface of Au–Ag nanorods via a 5′ end thiol group introduced into the structure of the fluorescein-labeled DNA aptamer sgc8c (Section 3.1). The avidity of the resulting aptamer-nanorods towards the tyrosine kinase 7 PTK7 transmembrane protein on CCFR-CEM cells was shown to be 26-fold higher than the affinity of the unconjugated fluorescein-labeled aptamer sgc8c for the same cells. The fluorescence intensity signal observed by flow cytometry was also 300-fold greater for the aptamer-nanorods labeled cells than the signals observed for CCFR-CEM cells labeled with the unconjugated fluorescein-labeled aptamer [70].

RNA aptamers directed at the prostate-specific membrane antigen (PSMA) have been used in the design of numerous nanostructures. Streptavidin-coated quantum dots (QD; semiconductor nanocrystals) have also been decorated with a biotinylated, 70-nucleotide long PSMA-specific RNA aptamer termed A9 and the resulting conjugates used for cellular imaging. Specifically, the photostability and small size of quantum dots was shown to improve the visualization of PSMA-positive cells (LNCaP) as adherent cell monolayers, in suspension preparations and embedded in a collagen matrix [71]. Aptamer particles have also been designed to serve the dual purpose of acting as a tumor-targeted agent and as a particle capable of controlled drug release. For example, the FITC-labeled PSMA-specific RNA aptamer A10 was coupled to a poly(lactic acid)-block-polyethylene glycol (PEG) co-polymer nanoparticles that have been derivatized with a terminal carboxylic acid functional group (PLA-PEG-COOH). Rhodamine-labeled dextran was encapsulated (as a model drug) into these polymeric

particles. The nanoparticles including their cargo were selectively imported into PSMA-positive LNCaP cells as confirmed by fluorescence microscopy [72]. Farokhzad et al. [73] subsequently loaded docetaxel, a chemotherapeutic drug into the aptamer-conjugated nanoparticles and injected a single intratumoral dose of the construct in nude mice harboring a LNCaP xenograft. Significant tumor regression was observed with no apparent immunogenicity. More recently, the same aptamer–nanoparticle conjugates were loaded with docetaxel and doxorubicin [74] or with cisplatin although the overall improvement in survival in the treated tumor-bearing animals was modest in relation to the non-aptamer-targeted drug loaded nanoparticles [75]. Finally, the creation of a conjugate composed of the PSMA-specific RNA aptamer A10–doxorubicin–quantum dot was recently reported by Jon and Farokhzad groups [76]. Again, this nanostructure is imported into $PSMA^+$ LNCaP prostate cancer cells by PSMA-mediated endocytosis. The construct offers the dual advantages of specifically delivering doxorubicin intercalated into the A10 aptamer structure to prostate cancer cells as well as imaging the delivery process through a FRET event arising from interactions of the released doxorubicin and the QD itself [76].

To date, liposomes remain one of the most successful drug delivery systems [77]. Liposome formulations of many of the most frequently prescribed chemotherapeutic drugs have been approved and are currently used in clinical practice [78]. Liposomes have been shown to increase the circulation time of aptamers while these aptamers aid in targeting liposomes to their desired site of action [73,79]. Liposomal drug delivery strategies have focused on developing long-circulating liposomes that target areas of increased vascular permeability via the enhanced permeation and retention (EPR) effect [80]. The EPR effect however remains a passive tumor localization strategy that can lead to detrimental systemic consequences and suboptimal antitumor efficacy [81,82]. Aptamer-labeled liposomes can thus increase the delivery of encapsulated therapeutic agents to cancer cells.

3.5. Challenges facing the in vivo use of aptamers

The concept of using aptamers as therapeutic agents was initially tested by selecting aptamers to thrombin with a view to preventing blood clotting [83]. The rationale for creating thrombin-selective aptamers was to generate heparin mimics that did not form complexes with platelet factor 4 which reacts with platelet-activating antibodies leading to heparin-induced thrombocytopenia [84]. Larry Gold's group selected aptamers against the targeted HIV reverse transcriptase [85]. Since virus transcriptases normally bind nucleic acids, they represent excellent aptamer targets. Other parts of the virus are also being targeted by aptamers, some of which are DNA aptamers [86,87]. In spite of their large therapeutic potential, aptamer drugs are still not a commonplace treatment mostly due to the previously mentioned challenges associated with translating small scale *in vitro* laboratory experiments into medical practice. Currently, the only aptamer approved by the FDA is Macugen (OSI Pharmaceuticals and Pfizer), an aptamer used to treat age-related macular degeneration (AMD). Macugen is a PEGylated 29-nucleotide long RNA aptamer with a modified backbone that significantly increases its circulating half-life [88]. Macugen recognizes the vascular endothelial growth factor isoform VEGF165 but does not bind to VEGF121 [88]. In contrast, the antibody against VEGF marketed by Genentech under the name Ranibizumab shows specificity towards both isoforms [89].

Aptamer structures can be evolved to recognize minor structural differences within a given target and typically bind to their targets with affinities comparable to those of antibodies [90,91]. Practical advantages of aptamers over antibodies include their lower mass, low cost of synthesis, long shelf-life and consistent quality. However, aptamers do face challenges as potential therapeutic or delivery agents. Firstly, nucleic acids are small, charged molecules. As such, they cannot passively traverse a cell membrane. Secondly, oligonucleotides are rapidly degraded by nucleases in plasma and cleared from circulation, resulting in short *in vivo* half-lives [67,92]. Thirdly, oligonucleotides are typically not immunogenic. Yet, immune responses mediated by Toll-like receptor family members have been reported as exemplified by unmethylated CpG sequences [93]. Solutions to these challenges are available. There are several approaches for increasing the circulating time (half-life) of aptamers in plasma. One of them is PEGylation, the process of conjugating polyethylene glycol (PEG) groups to such molecules. The coupling of a cholesterol group or a cell-penetrating peptide can also reduce their systemic clearance [79,94]. Another approach is by using chemically modified nucleotides shown to increase the half-life of aptamer sequences by more than 40-fold [95]. Such changes can be introduced during the SELEX process by using modified nucleotides that are incorporated by the T7 polymerase at the *in vitro* transcription step when RNA aptamers are being selected. In the case of DNA aptamers, modified nucleotides are simply introduced during library synthesis [96,97]. Possible modifications compatible with the SELEX protocol include substitution of the 2′ OH group with a 2′ fluoro or 2′ amino group [98,99]. Besides the sugar component of the molecule, various groups such as aromatic and alkyl moieties can be attached to the C5 position of UTP [100]. Other modifications termed "post SELEX" have been introduced after a useful sequence is identified [101]. One form of post SELEX modification is Locked Nucleic Acid (LNA) [102]. The LNAs can have one or more nucleotides with a methylene linkage between the 2′ oxygen and the 4′ carbon, which results in the "locked" conformation of the sugar. This modification provides an increased affinity for the complementary strand, higher thermal stability, and resistance to nuclease degradation [103]. Multivalency represents another factor that can increase the avidity and potency of aptamers, as demonstrated by the oligomerization of an RNA aptamer against the *Drosophila* protein B52 [104]. The tetravalent RNA aptamer recognizing the cytotoxic T-cell antigen-4 (CTLA-4) has also shown a therapeutic advantage over its monomeric counterpart in prolonging the survival of C57BL/6 mice implanted with the B16/F10.9 murine melanoma [105].

Among other aptamers selected to target tumor specific proteins, the first one to enter clinical trials is an unmodified DNA aptamer termed AS1411 (Antisoma). It was shown that its G rich sequence binds nucleolin present on the surface of cancer cells and can inhibit NF-κB pathways [106,107]. This aptamer is currently in Phase II clinical trials and shows activity towards many types of hematological cancers (clinical trials.gov identifier NCT00512083; NCT00740441). Interestingly, this 26-nucleotide long unmodified DNA aptamer is stable in serum, which indicates that the sequence of the aptamers results in a three-dimensional structure that is not easily susceptible to nuclease degradation [108]. Thus, the need to further modify DNA aptamers to increase their stability *in vivo* may not be necessary in all cases.

Finally, Fig. 6 outlines how aptamer-cargoes can reach several intracellular vesicular compartments. The illustration is also meant to highlight the fact that the cytosolic release of cargoes entrapped in vesicles remains an inefficient process and a common challenge confronting other drug delivery strategies involving polymer formulations, antibody conjugates and cell-penetrating peptides. Aptamer-targeted cargoes such as radionuclides (acting within a cell diameter or via a bystander effect), hydrophobic drugs, gold particles and liposomes may reach the cytosol or have their therapeutic effect enhanced by simply residing or cycling through vesicles. Other charged cargoes such as siRNAs, plasmids and proteins will be inefficiently released from endosomal compartments and may require the use of endosomolytic agents.

4. Concluding remarks

A major challenge associated with chemotherapeutic agents remains their toxicity towards normal tissues. This challenge limits

their use to suboptimal doses and ultimately leads to treatment failure. Therapeutic cargoes linked to antibodies are being developed to specifically deliver chemotherapeutic agents to cancer cells. Yet, antibody-guided therapies come with major limiting factors including their size, cost and immunogenicity. Accordingly, simpler targeting agents are needed to focus the delivery of useful cargoes to cancer cells. Since their inception in 1990 [1,2] short DNA/RNA aptamers have been developed to recognize therapeutically important molecular targets such as VEGF, thrombin and HIV. More importantly, aptamers can serve as cellular delivery vehicles by targeting cell surface markers that are internalized by cancer cells, allowing for the intracellular localization of therapeutic cargoes. Aptamers can be rapidly developed through SELEX screens, are easily synthesized, are typically non-immunogenic and are readily amenable to modifications leading to increased circulation times and stability. Aptamers directed at internalized surface markers can be conjugated directly to drugs, RNA/DNA, radionuclides, proteins and nanostructures to serve as tumor selective diagnostic and therapeutic agents.

Acknowledgements

We would like to thank Dr. Aaron Schimmer and Dr. Jeffrey Schlom for providing us with CD33-positive and CEA-positive cell lines respectively. We also wish to acknowledge the financial support of the Canadian Breast Cancer Research Alliance in association with the Canadian Cancer Society.

References

[1] A.D. Ellington, J.W. Szostak, In vitro selection of RNA molecules that bind specific ligands, Nature 346 (1990) 818–822.
[2] C. Tuerk, L. Gold, Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase, Science 249 (1990) 505–510.
[3] K. Padmanabhan, K.P. Padmanabhan, J.D. Ferrara, J.E. Sadler, A. Tulinsky, The structure of alpha-thrombin inhibited by a 15-mer single-stranded DNA aptamer, J. Biol. Chem. 268 (1993) 17651–17654.
[4] S.B. Long, M.B. Long, R.R. White, B.A. Sullenger, Crystal structure of an RNA aptamer bound to thrombin, RNA 14 (2008) 2504–2512.
[5] J.P. Sheehan, J.E. Sadler, Molecular mapping of the heparin-binding exosite of thrombin, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 5518–5522.
[6] W. Bode, D. Turk, A. Karshikov, The refined 1.9-A X-ray crystal structure of D-Phe-Pro-Arg chloromethylketone-inhibited human alpha-thrombin: structure analysis, overall structure, electrostatic properties, detailed active-site geometry, and structure–function relationships, Protein Sci. 1 (1992) 426–471.
[7] H. Grosjean, R. Benne, Modification and Editing of RNA, American Society for Microbiology, Washington DC, 1998.
[8] C.A. Wakeman, W.C. Winkler, C.E. Dann 3rd, Structural features of metabolite-sensing riboswitches, Trends Biochem. Sci. 32 (2007) 415–424.
[9] S.H. Chou, K.H. Chin, A.H. Wang, Unusual DNA duplex and hairpin motifs, Nucleic Acids Res. 31 (2003) 2461–2474.
[10] S. Neidle, G.N. Parkinson, The structure of telomeric DNA, Curr. Opin. Struct. Biol. 13 (2003) 275–283.
[11] A. Siddiqui-Jain, C.L. Grand, D.J. Bearss, L.H. Hurley, Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress c-MYC transcription, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 11593–11598.
[12] A.T. Phan, V. Kuryavyi, J.B. Ma, A. Faure, M.L. Andreola, D.J. Patel, An interlocked dimeric parallel-stranded DNA quadruplex: a potent inhibitor of HIV-1 integrase, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 634–639.
[13] Y. Teng, A.C. Girvan, L.K. Casson, W.M. Pierce Jr., M. Qian, S.D. Thomas, P.J. Bates, AS1411 alters the localization of a complex containing protein arginine methyltransferase 5 and nucleolin, Cancer Res. 67 (2007) 10491–10500.
[14] J. Liu, Z. Cao, Y. Lu, Functional nucleic acid sensors, Chem. Rev. 109 (2009) 1948–1998.
[15] S.K. Silverman, Deoxyribozymes: selection design and serendipity in the development of DNA catalysts, Acc. Chem. Res. 42 (2009) 1521–1531.
[16] S.S. Chang, V.E. Reuter, W.D. Heston, N.H. Bander, L.S. Grauer, P.B. Gaudin, Five different anti-prostate-specific membrane antigen (PSMA) antibodies confirm PSMA expression in tumor-associated neovasculature, Cancer Res. 59 (1999) 3192–3198.
[17] J.S. Ross, C.E. Sheehan, H.A. Fisher, R.P. Kaufman Jr., P. Kaur, K. Gray, I. Webb, G.S. Gray, R. Mosher, B.V. Kallakury, Correlation of primary tumor prostate-specific membrane antigen expression with disease recurrence in prostate cancer, Clin. Cancer Res. 9 (2003) 6357–6362.
[18] S.E. Lupold, B.J. Hicke, Y. Lin, D.S. Coffey, Identification and characterization of nuclease-stabilized RNA molecules that bind human prostate cancer cells via the prostate-specific membrane antigen, Cancer Res. 62 (2002) 4029–4033.
[19] H. Liu, A.K. Rajasekaran, P. Moy, Y. Xia, S. Kim, V. Navarro, R. Rahmati, N.H. Bander, Constitutive and antibody-induced internalization of prostate-specific membrane antigen, Cancer Res. 58 (1998) 4055–4060.
[20] S.L. Su, I.P. Huang, W.R. Fair, C.T. Powell, W.D. Heston, Alternatively spliced variants of prostate-specific membrane antigen RNA: ratio of expression as a potential measurement of progression, Cancer Res. 55 (1995) 1441–1443.
[21] J.O. McNamara 2nd, E.R. Andrechek, Y. Wang, K.D. Viles, R.E. Rempel, E. Gilboa, B.A. Sullenger, P.H. Giangrande, Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras, Nat. Biotechnol. 24 (2006) 1005–1015.
[22] S.D. Freeman, S. Kelm, E.K. Barber, P.R. Crocker, Characterization of CD33 as a new member of the sialoadhesin family of cellular interaction molecules, Blood 85 (1995) 2005–2012.
[23] R.G. Andrews, B. Torok-Storb, I.D. Bernstein, Myeloid-associated differentiation antigens on stem cells and their progeny identified by monoclonal antibodies, Blood 62 (1983) 124–132.
[24] R.G. Andrews, M. Takahashi, G.M. Segal, J.S. Powell, I.D. Bernstein, J.W. Singer, The L4F3 antigen is expressed by unipotent and multipotent colony-forming cells but not by their precursors, Blood 68 (1986) 1030–1035.
[25] J.D. Griffin, D. Linch, K. Sabbath, P. Larcom, S.F. Schlossman, A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells, Leuk. Res. 8 (1984) 521–534.
[26] P.A. Dinndorf, R.G. Andrews, D. Benjamin, D. Ridgway, L. Wolff, I.D. Bernstein, Expression of normal myeloid-associated antigens by acute leukemia cells, Blood 67 (1986) 1048–1053.
[27] D.A. Scheinberg, M. Tanimoto, S. McKenzie, A. Strife, L.J. Old, B.D. Clarkson, Monoclonal antibody M195: a diagnostic marker for acute myelogenous leukemia, Leukemia 3 (1989) 440–445.
[28] L.W. Terstappen, M. Safford, S. Konemann, M.R. Loken, K. Zurlutter, T. Buchner, W. Hiddemann, B. Wormann, Flow cytometric characterization of acute myeloid leukemia. Part II. Phenotypic heterogeneity at diagnosis, Leukemia 6 (1992) 70–80.
[29] M.C. Putti, R. Rondelli, M.G. Cocito, M. Arico, L. Sainati, V. Conter, C. Guglielmi, A. Cantu-Rajnoldi, R. Consolini, A. Pession, L. Zanesco, G. Masera, A. Biondi, G. Basso, Expression of myeloid markers lacks prognostic impact in children treated for acute lymphoblastic leukemia: Italian experience in AIEOP-ALL 88–91 studies, Blood 92 (1998) 795–801.
[30] I.D. Bernstein, Monoclonal antibodies to the myeloid stem cells: therapeutic implications of CMA-676, a humanized anti-CD33 antibody calicheamicin conjugate, Leukemia 14 (2000) 474–475.
[31] P.R. Hamann, L.M. Hinman, C.F. Beyer, D. Lindh, J. Upeslacis, D.A. Flowers, I. Bernstein, An anti-CD33 antibody–calicheamicin conjugate for treatment of acute myeloid leukemia. Choice of linker, Bioconjug. Chem. 13 (2002) 40–46.
[32] V.H. van Der Velden, J.G. te Marvelde, P.G. Hoogeveen, I.D. Bernstein, A.B. Houtsmuller, M.S. Berger, J.J. van Dongen, Targeting of the CD33-calicheamicin immunoconjugate Mylotarg (CMA-676) in acute myeloid leukemia: in vivo and in vitro saturation and internalization by leukemic and normal myeloid cells, Blood 97 (2001) 3197–3204.
[33] R.B. Walter, B.W. Raden, D.M. Kamikura, J.A. Cooper, I.D. Bernstein, Influence of CD33 expression levels and ITIM-dependent internalization on gemtuzumab ozogamicin-induced cytotoxicity, Blood 105 (2005) 1295–1302.
[34] A.M. Tsimberidou, F.J. Giles, E. Estey, S. O'Brien, M.J. Keating, H.M. Kantarjian, The role of gemtuzumab ozogamicin in acute leukaemia therapy, Br. J. Haematol. 132 (2006) 398–409.
[35] V.H. van der Velden, N. Boeckx, I. Jedema, J.G. te Marvelde, P.G. Hoogeveen, M. Boogaerts, J.J. van Dongen, High CD33-antigen loads in peripheral blood limit the efficacy of gemtuzumab ozogamicin (Mylotarg) treatment in acute myeloid leukemia patients, Leukemia 18 (2004) 983–988.
[36] P. Chevallier, N. Robillard, S. Ayari, T. Guillaume, J. Delaunay, F. Mechinaud, H. Avet-Loiseau, M. Mohty, J.L. Harousseau, R. Garand, Persistence of CD33 expression at relapse in CD33(+) acute myeloid leukaemia patients after receiving Gemtuzumab in the course of the disease, Br. J. Haematol. 143 (2008) 744–746.
[37] P. Chen, J. Wang, K. Hope, L. Jin, J. Dick, R. Cameron, J. Brandwein, M. Minden, R.M. Reilly, Nuclear localizing sequences promote nuclear translocation and enhance the radiotoxicity of the anti-CD33 monoclonal antibody HuM195 labeled with 111In in human myeloid leukemia cells, J. Nucl. Med. 47 (2006) 827–836.
[38] P. Gold, S.O. Freedman, Demonstration of tumor-specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques, J. Exp. Med. 121 (1965) 439–462.
[39] S. Hammarstrom, The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues, Semin. Cancer Biol. 9 (1999) 67–81.
[40] D. Boucher, D. Cournoyer, C.P. Stanners, A. Fuks, Studies on the control of gene expression of the carcinoembryonic antigen family in human tissue, Cancer Res. 49 (1989) 847–852.
[41] M.J. Goldstein, E.P. Mitchell, Carcinoembryonic antigen in the staging and follow-up of patients with colorectal cancer, Cancer Invest. 23 (2005) 338–351.
[42] L.B. Shih, D.M. Goldenberg, H. Xuan, H.W. Lu, M.J. Mattes, T.C. Hall, Internalization of an intact doxorubicin immunoconjugate, Cancer Immunol. Immunother. 38 (1994) 92–98.
[43] C.H. Ford, G.C. Tsaltas, P.A. Osborne, K. Addetia, Novel flow cytometric analysis of the progress and route of internalization of a monoclonal anti-carcinoembryonic antigen (CEA) antibody, Cytometry 23 (1996) 228–240.
[44] R. Stein, M. Juweid, M.J. Mattes, D.M. Goldenberg, Carcinoembryonic antigen as a target for radioimmunotherapy of human medullary thyroid carcinoma: antibody processing, targeting, and experimental therapy with 131I and 90Y labeled MAbs, Cancer Biother. Radiopharm. 14 (1999) 37–47.

[45] M.M. Schmidt, G.M. Thurber, K.D. Wittrup, Kinetics of anti-carcinoembryonic antigen antibody internalization: effects of affinity, bivalency, and stability, Cancer Immunol. Immunother. 57 (2008) 1879–1890.
[46] D.M. Goldenberg, Targeted therapy of cancer with radiolabeled antibodies, J. Nucl. Med. 43 (2002) 693–713.
[47] T.M. Behr, T. Liersch, L. Greiner-Bechert, F. Griesinger, M. Behe, P.M. Markus, S. Gratz, C. Angerstein, G. Brittinger, H. Becker, D.M. Goldenberg, W. Becker, Radioimmunotherapy of small-volume disease of metastatic colorectal cancer, Cancer 94 (2002) 1373–1381.
[48] G.P. Adams, R. Schier, A.M. McCall, H.H. Simmons, E.M. Horak, R.K. Alpaugh, J.D. Marks, L.M. Weiner, High affinity restricts the localization and tumor penetration of single-chain fv antibody molecules, Cancer Res. 61 (2001) 4750–4755.
[49] C.P. Graff, K.D. Wittrup, Theoretical analysis of antibody targeting of tumor spheroids: importance of dosage for penetration, and affinity for retention, Cancer Res. 63 (2003) 1288–1296.
[50] P.F. Robbins, J.A. Kantor, M. Salgaller, P.H. Hand, P.D. Fernsten, J. Schlom, Transduction and expression of the human carcinoembryonic antigen gene in a murine colon carcinoma cell line, Cancer Res. 51 (1991) 3657–3662.
[51] M.A. McGuckin, M.D. Walsh, B.G. Hohn, B.G. Ward, R.G. Wright, Prognostic significance of MUC1 epithelial mucin expression in breast cancer, Hum. Pathol. 26 (1995) 432–439.
[52] A.P. Spicer, G.J. Rowse, T.K. Lidner, S.J. Gendler, Delayed mammary tumor progression in Muc-1 null mice, J. Biol. Chem. 270 (1995) 30093–30101.
[53] J. Taylor-Papadimitriou, J. Burchell, D.W. Miles, M. Dalziel, MUC1 and cancer, Biochim. Biophys. Acta 1455 (1999) 301–313.
[54] F.G. Hanisch, S. Muller, MUC1: the polymorphic appearance of a human mucin, Glycobiology 10 (2000) 439–449.
[55] S. Gendler, J. Taylor-Papadimitriou, T. Duhig, J. Rothbard, J. Burchell, A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made up of tandem repeats, J. Biol. Chem. 263 (1988) 12820–12823.
[56] F.G. Hanisch, T.R. Stadie, F. Deutzmann, J. Peter-Katalinic, MUC1 glycoforms in breast cancer-cell line T47D as a model for carcinoma-associated alterations of 0-glycosylation, Eur. J. Biochem. 236 (1996) 318–327.
[57] K.O. Lloyd, J. Burchell, V. Kudryashov, B.W. Yin, J. Taylor-Papadimitriou, Comparison of O-linked carbohydrate chains in MUC-1 mucin from normal breast epithelial cell lines and breast carcinoma cell lines. Demonstration of simpler and fewer glycan chains in tumor cells, J. Biol. Chem. 271 (1996) 33325–33334.
[58] S.V. Litvinov, J. Hilkens, The epithelial sialomucin, episialin, is sialylated during recycling, J. Biol. Chem. 268 (1993) 21364–21371.
[59] R.L. Ceriani, C.M. Chan, F.S. Baratta, L. Ozzello, C.M. DeRosa, D.V. Habif, Levels of expression of breast epithelial mucin detected by monoclonal antibody BrE-3 in breast-cancer prognosis, Int. J. Cancer 51 (1992) 343–354.
[60] Y. Altschuler, C.L. Kinlough, P.A. Poland, J.B. Bruns, G. Apodaca, O.A. Weisz, R.P. Hughey, Clathrin-mediated endocytosis of MUC1 is modulated by its glycosylation state, Mol. Biol. Cell 11 (2000) 819–831.
[61] P. Henderikx, N. Coolen-van Neer, A. Jacobs, E. van der Linden, J.W. Arends, J. Mullberg, H.R. Hoogenboom, A human immunoglobulin G1 antibody originating from an in vitro-selected Fab phage antibody binds avidly to tumor-associated MUC1 and is efficiently internalized, Am. J. Pathol. 160 (2002) 1597–1608.
[62] C.S. Ferreira, M.C. Cheung, S. Missailidis, S. Bisland, J. Gariepy, Phototoxic aptamers selectively enter and kill epithelial cancer cells, Nucleic Acids Res. 37 (2009) 866–876.
[63] Y.F. Huang, D. Shangguan, H. Liu, J.A. Phillips, X. Zhang, Y. Chen, W. Tan, Molecular assembly of an aptamer–drug conjugate for targeted drug delivery to tumor cells, Chembiochem 10 (2009) 862–868.
[64] V. Bagalkot, O.C. Farokhzad, R. Langer, S. Jon, An aptamer–doxorubicin physical conjugate as a novel targeted drug-delivery platform, Angew. Chem. Int. Ed. Engl. 45 (2006) 8149–8152.
[65] D. Willner, P.A. Trail, S.J. Hofstead, H.D. King, S.J. Lasch, G.R. Braslawsky, R.S. Greenfield, T. Kaneko, R.A. Firestone, (6-Maleimidocaproyl)hydrazone of doxorubicin—a new derivative for the preparation of immunoconjugates of doxorubicin, Bioconjug. Chem. 4 (1993) 521–527.
[66] V.S. Goldmacher, C.F. Scott, J.M. Lambert, G.D. McIntyre, W.A. Blattler, A.R. Collnhson, J.K. Stewart, L.D. Chong, S. Cook, H.S. Slayter, et al., Cytotoxicity of gelonin and its conjugates with antibodies is determined by the extent of their endocytosis, J. Cell. Physiol. 141 (1989) 222–234.
[67] T.C. Chu, J.W. Marks 3rd, L.A. Lavery, S. Faulkner, M.G. Rosenblum, A.D. Ellington, M. Levy, Aptamer:toxin conjugates that specifically target prostate tumor cells, Cancer Res. 66 (2006) 5989–5992.
[68] B.J. Hicke, A.W. Stephens, T. Gould, Y.F. Chang, C.K. Lynott, J. Heil, S. Borkowski, C.S. Hilger, G. Cook, S. Warren, P.G. Schmidt, Tumor targeting by an aptamer, J. Nucl. Med. 47 (2006) 668–678.
[69] B.J. Hicke, C. Marion, Y.F. Chang, T. Gould, C.K. Lynott, D. Parma, P.G. Schmidt, S. Warren, Tenascin-C aptamers are generated using tumor cells and purified protein, J. Biol. Chem. 276 (2001) 48644–48654.
[70] Y.F. Huang, H.T. Chang, W. Tan, Cancer cell targeting using multiple aptamers conjugated on nanorods, Anal. Chem. 80 (2008) 567–572.
[71] T.C. Chu, F. Shieh, L.A. Lavery, M. Levy, R. Richards-Kortum, B.A. Korgel, A.D. Ellington, Labeling tumor cells with fluorescent nanocrystal–aptamer bioconjugates, Biosens. Bioelectron. 21 (2006) 1859–1866.
[72] O.C. Farokhzad, S. Jon, A. Khademhosseini, T.N. Tran, D.A. Lavan, R. Langer, Nanoparticle–aptamer bioconjugates: a new approach for targeting prostate cancer cells, Cancer Res. 64 (2004) 7668–7672.
[73] O.C. Farokhzad, J. Cheng, B.A. Teply, I. Sherifi, S. Jon, P.W. Kantoff, J.P. Richie, R. Langer, Targeted nanoparticle–aptamer bioconjugates for cancer chemotherapy in vivo, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 6315–6320.
[74] L. Zhang, A.F. Radovic-Moreno, F. Alexis, F.X. Gu, P.A. Basto, V. Bagalkot, S. Jon, R.S. Langer, O.C. Farokhzad, Co-delivery of hydrophobic and hydrophilic drugs from nanoparticle–aptamer bioconjugates, Chem. Med. Chem. 2 (2007) 1268–1271.
[75] S. Dhar, F.X. Gu, R. Langer, O.C. Farokhzad, S.J. Lippard, Targeted delivery of cisplatin to prostate cancer cells by aptamer functionalized Pt(IV) prodrug-PLGA-PEG nanoparticles, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 17356–17361.
[76] V. Bagalkot, L. Zhang, E. Levy-Nissenbaum, S. Jon, P.W. Kantoff, R. Langer, O.C. Farokhzad, Quantum dot–aptamer conjugates for synchronous cancer imaging, therapy, and sensing of drug delivery based on bi-fluorescence resonance energy transfer, Nano Lett. 7 (2007) 3065–3070.
[77] Y. Kaneda, Virosomes: evolution of the liposome as a targeted drug delivery system, Adv. Drug Deliv. Rev. 43 (2000) 197–205.
[78] A. Wagner, M. Platzgummer, G. Kreismayr, H. Quendler, G. Stiegler, B. Ferko, G. Vecera, K. Vorauer-Uhl, H. Katinger, GMP production of liposomes—a new industrial approach, J. Liposome Res. 16 (2006) 311–319.
[79] M.C. Willis, B.D. Collins, T. Zhang, L.S. Green, D.P. Sebesta, C. Bell, E. Kellogg, S.C. Gill, A. Magallanez, S. Knauer, R.A. Bendele, P.S. Gill, N. Janjic, Liposome-anchored vascular endothelial growth factor aptamers, Bioconjug. Chem. 9 (1998) 573–582.
[80] Y. Matsumura, H. Maeda, A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs, Cancer Res. 46 (1986) 6387–6392.
[81] C. Mamot, D.C. Drummond, U. Greiser, K. Hong, D.B. Kirpotin, J.D. Marks, J.W. Park, Epidermal growth factor receptor (EGFR)-targeted immunoliposomes mediate specific and efficient drug delivery to EGFR- and EGFRvIII-overexpressing tumor cells, Cancer Res. 63 (2003) 3154–3161.
[82] J. Woo, G.N. Chiu, G. Karlsson, E. Wasan, L. Ickenstein, K. Edwards, M.B. Bally, Use of a passive equilibration methodology to encapsulate cisplatin into preformed thermosensitive liposomes, Int. J. Pharm. 349 (2008) 38–46.
[83] L.C. Bock, L.C. Griffin, J.A. Latham, E.H. Vermaas, J.J. Toole, Selection of single-stranded DNA molecules that bind and inhibit human thrombin, Nature 355 (1992) 564–566.
[84] W.K. Stribling, T.F. Slaughter, T.T. Houle, D.C. Sane, Beyond the platelet count: heparin antibodies as independent risk predictors, Am. Heart J. 153 (2007) 900–906.
[85] D.J. Schneider, J. Feigon, Z. Hostomsky, L. Gold, High-affinity ssDNA inhibitors of the reverse transcriptase of type 1 human immunodeficiency virus, Biochemistry 34 (1995) 9599–9610.
[86] S.H. Chou, K.H. Chin, A.H. Wang, DNA aptamers as potential anti-HIV agents, Trends Biochem. Sci. 30 (2005) 231–234.
[87] J. Zhou, P. Swiderski, H. Li, J. Zhang, C.P. Neff, R. Akkina, J.J. Rossi, Selection, characterization and application of new RNA HIV gp 120 aptamers for facile delivery of Dicer substrate siRNAs into HIV infected cells, Nucleic Acids Res. 37 (2009) 3094–3109.
[88] E.W. Ng, D.T. Shima, P. Calias, E.T. Cunningham Jr., D.R. Guyer, A.P. Adamis, Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease, Nat. Rev. Drug Discov. 5 (2006) 123–132.
[89] H. Kourlas, P. Abrams, Ranibizumab for the treatment of neovascular age-related macular degeneration: a review, Clin. Ther. 29 (2007) 1850–1861.
[90] R. Conrad, L.M. Keranen, A.D. Ellington, A.C. Newton, Isozyme-specific inhibition of protein kinase C by RNA aptamers, J. Biol. Chem. 269 (1994) 32051–32054.
[91] D.J. Schneider, J. Feigon, Z. Hostomsky, L. Gold, High-affinity ssDNA inhibitors of the reverse transcriptase of type 1 human immunodeficiency virus, Biochemistry 34 (1995) 9599–9610.
[92] R.R. White, B.A. Sullenger, C.P. Rusconi, Developing aptamers into therapeutics, J. Clin. Invest. 106 (2000) 929–934.
[93] H. Wagner, The immunobiology of the TLR9 subfamily, Trends Immunol. 25 (2004) 381–386.
[94] J.M. Healy, S.D. Lewis, M. Kurz, R.M. Boomer, K.M. Thompson, C. Wilson, T.G. McCauley, Pharmacokinetics and biodistribution of novel aptamer compositions, Pharm. Res. 21 (2004) 2234–2246.
[95] C.G. Peng, M.J. Damha, G-quadruplex induced stabilization by 2′-deoxy-2′-fluoro-D-arabinonucleic acids (2′F-ANA), Nucleic Acids Res. 35 (2007) 4977–4988.
[96] H. Aurup, D.M. Williams, F. Eckstein, 2′-Fluoro- and 2′-amino-2′-deoxynucleoside 5′-triphosphates as substrates for T7 RNA polymerase, Biochemistry 31 (1992) 9636–9641.
[97] J.A. Latham, R. Johnson, J.J. Toole, The application of a modified nucleotide in aptamer selection: novel thrombin aptamers containing 5-(1-pentynyl)-2′-deoxyuridine, Nucleic Acids Res. 22 (1994) 2817–2822.
[98] R. Padilla, R. Sousa, Efficient synthesis of nucleic acids heavily modified with non-canonical ribose 2′-groups using a mutantT7 RNA polymerase (RNAP), Nucleic Acids Res. 27 (1999) 1561–1563.
[99] D. Jellinek, L.S. Green, C. Bell, C.K. Lynott, N. Gill, C. Vargeese, G. Kirschenheuter, D.P. McGee, P. Abesinghe, W.A. Pieken, et al., Potent 2′-amino-2′-deoxypyrimidine RNA inhibitors of basic fibroblast growth factor, Biochemistry 34 (1995) 11363–11372.
[100] T. Schoetzau, J. Langner, E. Moyroud, I. Roehl, S. Vonhoff, S. Klussmann, Amino-modified nucleobases: functionalized nucleoside triphosphates applicable for SELEX, Bioconjug. Chem. 14 (2003) 919–926.
[101] B.E. Eaton, L. Gold, B.J. Hicke, N. Janjic, F.M. Jucker, D.P. Sebesta, T.M. Tarasow, M.C. Willis, D.A. Zichi, Post-SELEX combinatorial optimization of aptamers, Bioorg. Med. Chem. 5 (1997) 1087–1096.
[102] J. Barciszewski, M. Medgaard, T. Koch, J. Kurreck, V.A. Erdmann, Locked nucleic acid aptamers, Methods Mol. Biol. 535 (2009) 165–186.
[103] B. Vester, J. Wengel, LNA (locked nucleic acid): high-affinity targeting of complementary RNA and DNA, Biochemistry 43 (2004) 13233–13241.
[104] S. Santulli-Marotto, S.K. Nair, C. Rusconi, B. Sullenger, E. Gilboa, Multivalent RNA aptamers that inhibit CTLA-4 and enhance tumor immunity, Cancer Res. 63 (2003) 7483–7489.

[105] J.O. McNamara, D. Kolonias, F. Pastor, R.S. Mittler, L. Chen, P.H. Giangrande, B. Sullenger, E. Gilboa, Multivalent 4-1BB binding aptamers costimulate CD8+ T cells and inhibit tumor growth in mice, J. Clin. Invest. 118 (2008) 376–386.
[106] P.J. Bates, J.B. Kahlon, S.D. Thomas, J.O. Trent, D.M. Miller, Antiproliferative activity of G-rich oligonucleotides correlates with protein binding, J. Biol. Chem. 274 (1999) 26369–26377.
[107] A.C. Girvan, Y. Teng, L.K. Casson, S.D. Thomas, S. Juliger, M.W. Ball, J.B. Klein, W.M. Pierce Jr., S.S. Barve, P.J. Bates, AGRO100 inhibits activation of nuclear factor-kappaB (NF-kappaB) by forming a complex with NF-kappaB essential modulator (NEMO) and nucleolin, Mol. Cancer Ther. 5 (2006) 1790–1799.
[108] V. Dapic, P.J. Bates, J.O. Trent, A. Rodger, S.D. Thomas, D.M. Miller, Antiproliferative activity of G-quartet-forming oligonucleotides with backbone and sugar modifications, Biochemistry 41 (2002) 3676–3685.
[109] G.J. Tong, S.C. Hsiao, Z.M. Carrico, M.B. Francis, Viral capsid DNA aptamer conjugates as multivalent cell-targeting vehicles, J. Am. Chem. Soc. 131 (2009) 11174–11178.
[110] J. Cheng, B.A. Teply, I. Sherifi, J. Sung, G. Luther, F.X. Gu, E. Levy-Nissenbaum, A.F. Radovic-Moreno, R. Langer, O.C. Farokhzad, Formulation of functionalized PLGA-PEG nanoparticles for in vivo targeted drug delivery, Biomaterials 28 (2007) 869–876.
[111] Z. Cao, R. Tong, A. Mishra, W. Xu, G.C. Wong, J. Cheng, Y. Lu, Reversible cell-specific drug delivery with aptamer-functionalized liposomes, Angew. Chem. Int. Ed. Engl. 48 (2009) 6494–6498.
[112] T.C. Chu, F. Shieh, L.A. Lavery, M. Levy, R. Richards-Kortum, B.A. Korgel, A.D. Ellington, Labeling tumor cells with fluorescent nanocrystal-aptamer bioconjugates, Biosensors & Bioelectronics 21 (2006) 1859–1866.