Chapter 10
Cell-Specific Aptamers for Nano-medical Applications

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Abstract This chapter describes cell-type specific aptamers and their use in diagnostics and therapy. Aptamers are single-stranded oligo(deoxy)nucleotides that selectively bind to their target molecules with high affinity. Cell-type specific aptamers in particular can be identified via SELEX using isolated surface proteins or whole cells as targets.

Cell-type specific aptamers have been mostly selected targeting cancer cells, which essentially take into account that about 20 % of the deaths worldwide are due to cancer and cancer-related diseases. In the early stages of cancer, circulating cancer cells are very rare. Cancer cell-targeting aptamers allow the identification of these rare circulating cells, thereby providing new tools for early cancer detection and diagnosis. Furthermore, they can be easily synthesized with a variety of modifications. In this regard tumor cell-targeting aptamers are employed as potential delivery vehicles, whereas they are equipped with various cargo molecules, such as toxins, chemotherapeutics, or siRNA molecules, that allow for the development of cell-specific treatment regimens and the decrease of unwanted side effects.

Contents

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10.1 Cell-Specific Aptamers

Aptamers were first described in 1990 by Ellington and Szostak (1990) and Tuerk and Gold (1990a). Since then they have become an invaluable research tool and demonstrated a broad application prospected in fundamental research, drug development, clinical diagnosis, and therapy. Basically, aptamers (Latin *aptus* fitting; Greek *meros* particle) are single-chained oligo(deoxy)nucleotides that selectively bind to respective target molecules with high affinity. They form complex 3D structures, which may include stems, loops, bulges, hairpins, pseudoknots, junctions and quadruplexes, or combinations thereof (Stoltenburg et al. 2007; Cruz and Westhof 2011). The conformation of aptamers is often adapted to their specific targets, and selective binding is mediated by structure compatibility, stacking of aromatic rings, electrostatic and van der Waals interactions, hydrogen bonds, or from a combination of these (Hermann 2000). The identification of aptamers is achieved through an iterative process termed systematic evolution of ligands by exponential enrichment (SELEX). In general, four main steps are repeated several times: (a) incubation of a oligo(deoxy)nucleotide library with the target molecule, (b) partitioning of unbound from bound nucleic acids, (c) elution of the binder, and (d) amplification and generation of single-stranded oligo(deoxy) nucleotides (Fig. 10.1).

To date, diverse target structures, such as small molecules [e.g., ethanolamine (Mann et al. 2005)], antibiotics [e.g., neomycin (Wallis et al. 1995)], peptides, and proteins [e.g., APC (Müller et al. 2009)], were used as ligands in SELEX approaches. To increase their chemical diversity and stability, aptamers can be modified before or after the SELEX process. Modifications at the phosphate-ribose backbone or at the nucleobases (Orr 2001; Bouchard et al. 2010) are known, for example, the substitution of the $2'$-position with fluoride or methoxy groups (Bouchard et al. 2010). The recent years has seen a tremendous increase of aptamers that target a distinct or a series of cells and cell subtypes (Table 10.1). These aptamers have gained emerging interest since they are applicable and adaptable to various biomedical applications. Especially, the fabrication of nanodevices seems to be a promising task using cell-type-selective aptamers. Aptamers targeting distinct cells can be identified by different approaches. The most convenient one seems to be the direct use of a target cell during an in vitro selection process. Several studies describe cell-SELEX protocols (Raddatz et al. 2008; Cerchia et al. 2009). However, the molecular target of such approaches needs to be determined post-selectively (Shamah et al. 2008; Meyer et al. 2011).
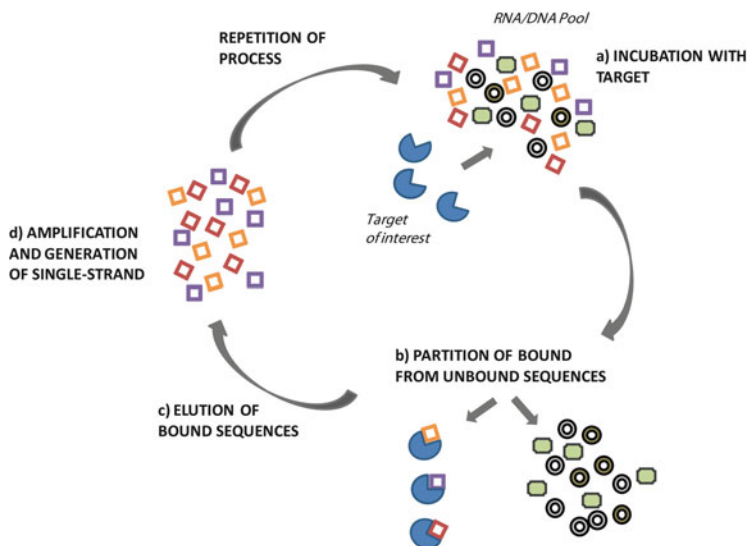
Fig. 10.1 Systematic evolution of ligands by exponential enrichment (SELEX) is used to select aptamers. This multistep process, composed of (a) incubation of the starting RNA or DNA pool with the target of interest, (b) partitioning of unbound from bound nucleic acids, (c) elution of the binder from the target, and (d) its amplification and single-strand generation, is repeated several times until an enriched population of aptamers is evolved

A target-driven approach can be achieved by recombinant expression of extracellular domains of cell-type-specific receptors or transmembrane proteins. Aptamers that have been generated against those proteins, through so-called classical SELEX protocols, need then to be analyzed afterwards regarding their potential to recognize the target when embedded in its natural environment. Also a combination of both approaches has been described (Hicke 2001). Alternatively, the target molecule can be expressed in cells that do not naturally bear the designated receptor. These cells can be used for selection experiments, whereas the non-expressing cell might be suitable for pre- or counterselection steps.

The first example of complex targets used in SELEX experiments was published in 1998 (Morris et al. 1998). In that membrane, preparations of red blood cells (RBC), so-called RBC ghosts, were addressed, and aptamers were enriched targeting two distinct membrane proteins. Besides, Morris and coworkers were able to isolate one of the aptamer's targets, namely, transferrin receptor monomer (CD71), by specific aptamer-based cross-linking experiments.

Since then, many cell-SELEX strategies were described, which are improved and optimized regarding minimization of high background binding of nucleic acids to the cell surface (Cerchia et al. 2005; Ohuchi et al. 2006). In addition, different cell-SELEX methods can be used to gain the desired biological activity of the aptamer. Our group reported an elegant, albeit elaborated, method to monitor the enrichment of putative cell-specific binder in 2008 (Raddatz et al. 2008). Here,

Table 10.1 List of complex-target-specific aptamers

Target	DNA/RNA	References
Viruses		
HIV	RNA	Tuerk et al. (1992)
RSV	RNA	Pan et al. (1995)
HPV16	RNA	Nicol et al. (2011)
Bacteria		
Anthrax spores	DNA	Bruno and Kiel (1999)
Campylobacter jejuni	DNA	Dwivedi et al. (2010)
M-type *Streptococcus pyogenes*	DNA	Hamula et al. (2011)
Pseudomonas aeruginosa	DNA	Wang et al. (2010)
Parasites		
Trypanosome brucei	RNA	Homann and Göringer (1999)
Trypanosome cruzi	RNA	Ulrich (2002)
Mammalian cells		
Pigpen endothelial cells	DNA	Blank (2001)
Murine pheochromocytoma cells	RNA	Cerchia et al. (2005)
Human red blood cells	DNA	Morris et al. (1998)
Human glioblastoma cells	RNA	Hicke (2001)
Human prostate cancer cells	RNA	Lupold et al. (2002)
Human B cells	DNA	Wu et al. (2003)
Human breast cancer cells	RNA	Chen (2003)
Human glioma cells	RNA	Cerchia et al. (2009)
Human epidermoid carcinoma	RNA	Li et al. (2011)

fluorescent-activated cell sorting (FACS) was implemented into the selection process to separate bound from unbound nucleic acids and, more importantly, dead from living cells. Dead cells are prone to nonspecifically take up nucleic acids what is counteracting on the enrichment process. In 2006, Ohuchi et al. published the so-called target expressed on cell surface-SELEX (TECS-SELEX) (Ohuchi et al. 2006). Here, the target of interest, namely, transforming growth factor-β type III receptor (TGF-β III receptor), was expressed on Chinese hamster ovary (CHO) cells, and thereby specific RNA aptamers were enriched. Furthermore, Cerchia et al. (2005) showed that a negative- or counter-SELEX step using a non-mutated or mutated target-expressing cell line captures unspecific binders. Cerchia and coworkers obtained rearranged during transfection (RET) receptor tyrosine kinase-specific RNA aptamers by using murine pheochromocytoma (PC12) cells, which express either no RET or different domains of RET. Thus, they drive the selection towards the human RET receptor mutated in the extracellular domain, which is published to be involved in multiple endocrine neoplasia syndrome 2A and familial medullary thyroid carcinoma (Jhiang 2000). Based on their investigations in 2005, Cerchia and coworkers proceed in whole-cell SELEX: in 2009, they generated aptamers, which specifically discriminate cells within the same tumor type (Cerchia et al. 2009). The clinical outcome of cancer is often hardly to tell, because of the heterogeneity of malignant cells. Cerchia et al. (2009) enriched aptamers that discriminate human U87MG glioma cells from less

malignant T98G cells and path the way for individual-specific cancer treatment. In 2001 Hicke et al. published a crossover approach: first, they performed nine rounds of selection-targeting tenascin-C-expressing U251 glioblastoma cells and then further enriched the sequences by incubation for two additional selection rounds with purified tenascin-C protein (Hicke 2001).

The significant step further in generating tumor-specific aptamers was described recently as a sole in vivo approach. Here, the physiological target conformation, conditioned by the target's microenvironment, is not impaired, and, thus, the naturally in situ condition of the target molecule is maintained. Mi et al. described a first in vivo selection experiment in 2009 (Mi et al. 2009). They focused on a mouse model, which bears intrahepatical colorectal cancer metastases. The starting modified RNA pool was administered intravenously into the tail vain. After circulation the mice were sacrificed and the tumor tissue resected. Subsequently, the tumor-associated RNA molecules were isolated, amplified, and prepared for the next in vivo selection cycle. In this way, a tumor-specific aptamer was identified after 14 rounds of selection. Further investigations revealed that the aptamer targets the p68 RNA helicase, which has been previously reported to be overexpressed on colorectal tumors (Abdelhaleem 2005). Cell-surface proteins represent accessible targets for developing novel therapies and diagnostics. The importance of potential cell-surface binders is shown by the fact that a large number of human diseases are linked to alterations maintained in these proteins (Sanders and Myers 2004; Josic et al. 2008). Aptamers that specifically target cell-surface molecules therefore have the potential to serve as therapeutic agonists, antagonists, or diagnostic agents. Selective aptamers may block the proliferation or metastatic induction of cell-surface receptors, either by acting directly on these or through the delivery of conjugated toxins to explicitly decimate tumor cell populations. They are also useful as imaging tools for diseases or stage-specific markers in diagnostics. By discrimination between cell types, aptamers allow the enrichment and purification of stem cells (Guo et al. 2006a) or the visualization of differentiated and non-differentiated cells (Berezovski et al. 2008). Studies in mammals revealed low immunogenicity and toxicity of aptamers, which is of utmost importance to conduct conclusive animal studies or applications in humans in later developmental stages. For example, Drolet et al. (2000) demonstrate the absence of severe side effects after administration of the anti-vascular endothelial growth factor (VEGF) aptamer NX1838, later known as EYE001 or macugen®, to rhesus monkeys. In addition, the administration of 1,000-fold higher doses of aptamers than required clinically immunogenicity was found to be absent or limited (Drolet et al. 2000; Eyetech Study Group 2002). In comparison to humanized antibodies, aptamers are devoid of residual sequences from other species and therefore are not prone to elicit human immune response. In the following sections, the applications of cell-specific aptamers in diagnostic and therapeutic settings will be discussed.

10.2   Aptamers in Cellular Diagnostics

Cell-specific aptamers have been mostly selected for cancer cells, which mainly takes into account that about 20 % of the deaths worldwide are due to cancer and cancer-related diseases (Alberts et al. 2008; Kumar et al. 2007). Cancer arises from a single cell, in which genetic or epigenetic changes occurred, resulting in excessive and unregulated proliferation independent of physiologic growth stimuli (Alberts et al. 2008; Kumar et al. 2007). However, transformation from healthy to cancer cells requires a number of independent mutations in proto-oncogenes and tumor suppressor genes (Alberts et al. 2008). Such mutations may occur in the germline or in somatic cells (Alberts et al. 2008). Germline mutations result in a hereditary predisposition to cancer, whereas mutations in somatic cells contribute to sporadic tumors (Alberts et al. 2008; Kumar et al. 2007; Knudson 2002). Overall, there are more than 100 distinct types of cancers, arising from various underlying molecular mechanisms (http://www.who.int/mediacentre/factsheets/fs297/en/). Early detection of cancer is thought to considerably increase the success of available treatment regimens. Therefore, comprehensive diagnosis is performed to detect cancer, even long before symptoms may occur (http://www.who.int/cancer/detection/en/). In this way preventive diagnostic approaches are available for breast cancer, cervical carcinoma, and colorectal cancer (http://www.who.int/cancer/detection/variouscancer/en/index.html). Early-stage cancer sees less than 100 circulating cancer cells in 1 ml of patient blood, which also bears approximately 5×10^9 other cells (Cristofanilli et al. 2004). This illustrates that diagnostic assays are required to specifically and with utmost sensitivity detect cancer cells. Commonly, diagnostic assays shall fulfill some prerequisites, such as robustness, sensitivity, and affordability. Cell-specific aptamers have entered the stage and proven to be well suited for diagnostic applications. Since aptamers are oligonucleotides, which fold into a specific three-dimensional structure, they can be denatured and refolded (Ellington and Szostak 1990). Hence, their binding is reversible; diagnostic assays based on aptamers can thus be recycled (Tuerk and Gold 1990b). The major challenge in aptamer-based biosensors is to convert the binding event of an aptamer to its target into a detectable signal (Fig. 10.2a). A multitude of different ways have been employed to achieve this conversion. Examples are fluorophores; nanoparticles, in particular, gold or iron; and quantum dots which were developed for detection purposes (Fig. 10.2b). Overall, aptamer-based diagnosis can be distinguished into two different setups: in solution or on chip (Fig. 10.2c).

10.2.1   Detection of Tumor Cells with Aptamers by Fluorescence Imaging

Fluorescence-based detection is the most common type of imaging in biological and medical applications (López-Colón et al. 2011). Aptamers can be easily
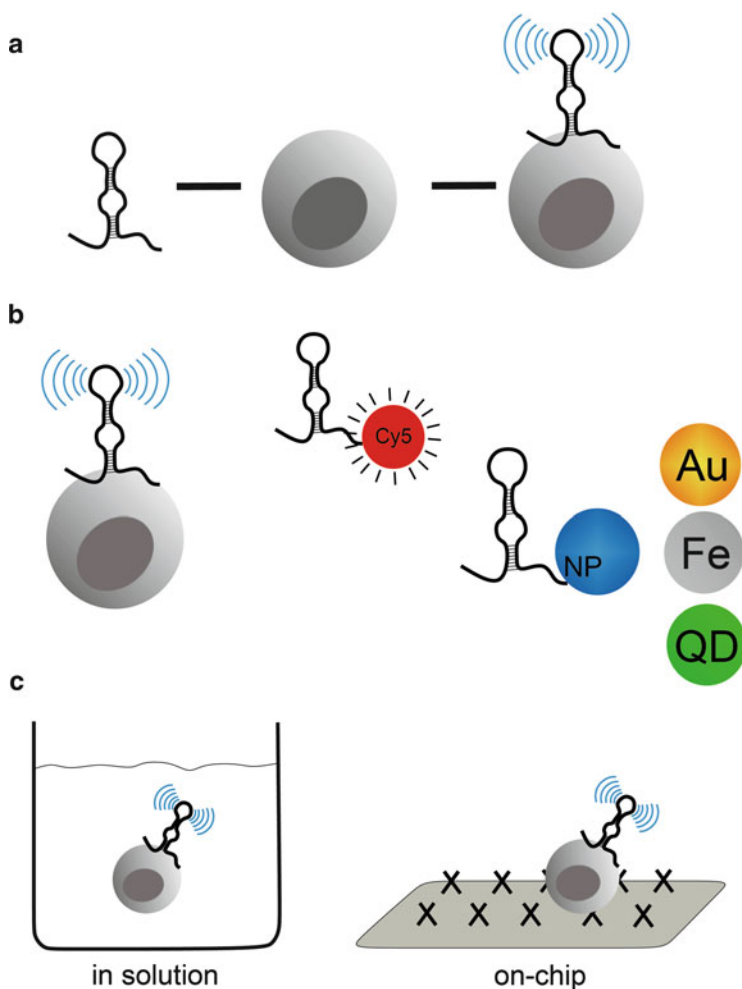
Fig. 10.2 Aptamers are selected either against a known specific biomarker on target cells or against an unknown receptor on the cell surface. (**a**) The binding of cell aptamers needs to be converted into a detectable signal. (**b**) A multitude of different ways have been employed to achieve this conversion, such as fluorophores; nanoparticles, in particular, gold (Au) or iron (Fe); and quantum dots (QD). (**c**) Aptamers have been mainly used in two different setups either in solution or on-chip

modified with fluorescent dyes. Upon target recognition the fluorescent signal can be detected by various fluorescence imaging systems, offering different resolutions depending on the employed fluorophore and overall aptamer performance (Weissleder and Ntziachristos 2003; Weissleder and Pittet 2008). By this means, aptamer–cell interactions can be validated by fluorescently labeled aptamers and cytometry, fluorescence, or confocal microscopy (Shangguan et al. 2006, 2008;

Tang et al. 2007; Sefah et al. 2009). Labeled aptamers targeting leukemia cells, such as acute T-cell leukemia (CEM), Burkitt's lymphoma (Ramos), and non-Hodgkin's B-cell lymphoma (Toledo), were shown to allow specific detection of leukemia cells in patient samples (Shangguan et al. 2006; Tang et al. 2007; Sefah et al. 2009). Even though significant progress has been made in ex vivo applications, aptamer-based in vivo noninvasive imaging is still quite rare. In vivo imaging is essential to localize primary tumor sites and, more importantly, metastases. Visualization is not only relevant for initial detection of tumors, but it is furthermore implicated when controlling tumor regression during therapy or in recognizing cancer relapse. In comparison to relatively large antibodies, aptamers are excellently suited for in vivo applications. Their small size facilitates rapid diffusion into the targeted tissues and interaction with the target molecules. However, aptamer half-life in blood is rather short, due to nuclease degradation, renal excretion, and hepatobiliary clearance (Charlton et al. 1997; Dougan et al. 2003). The circulation time of aptamers can be increased by chemical modifications, such as the introduction of locked nucleic acids (LNAs) or by polyethylene glycol (PEG) (Kurreck 2002; Healy et al. 2004). TD05, an aptamer selected against Burkitt's lymphoma cells, was successfully tested by this means in a xenograft nude mice model (Shi et al. 2010).

Fluorophore-based assays have disadvantages, resulting in a need for different detection methods. Biosensors employing optical signals are prone to background interference in complex biological samples, resulting in a reduction of detection capability. Therefore, other signaling moieties are employed, namely, nanoparticles.

10.2.2 Nanoparticles

Nanoparticles (NPs) are chemically designed materials sizing from 5 to 200 nm, which are used in various biomedical applications (Shi et al. 2010). Several fabrications of NPs are yet available with varying properties but adaptable to aptamer technology.

Quantum dots (QDs) are single crystals with only a few nanometers diameter. Their dimension can be controlled by applying defined temperature, duration, and presence of ligands during synthesis protocols (Jaiswal et al. 2002; Michalet 2005). QDs have several advantages over fluorophores; they show higher photostability, increased brightness, and narrow fluorescent spectra (Murray et al. 1993; Alivisatos 1996; Peng et al. 2000). Cell-targeting aptamers equipped with QDs have been used in cell imaging approaches. Prostate cancer is the second leading cause of cancer death in American man (Greenlee et al. 2001). In 2002, Lupold et al. selected two aptamers, A9 and A10, against the PSMA-expressing prostate tumor cell line LNCaP (Lupold et al. 2002). A9 was modified with QDs to specifically detect and label prostate cancer cells (Chu et al. 2006a). In this study, the aptamers were biotinylated and coupled to the streptavidin-coated quantum dots by

biotin–streptavidin interaction. The A9 aptamer:QD conjugates specifically recognized PSMA on live and fixed cells (Chu et al. 2006a). Bagalkot et al. (2007) took the next step by constructing a QD equipped with the PSMA aptamer to synchronously visualize and treat cancer cells in vitro (Section 10.3.1).

Gold nanoparticles (AuNPs) can be employed in reflectance imaging, since a single gold nanoparticle produces a signal, which is 10^6 times stronger than the signal generated by organic fluorophores (Smith et al. 2007; Bamrungsap et al. 2012). Furthermore, AuNPs are not prone to photo-bleaching opposed to organic dyes. For these reasons, cell-targeting aptamers have been functionalized via thiol chemistry with gold nanoparticles for in vitro imaging purposes. AuNPs were equipped with an aptamer towards platelet-derived growth factor (PDGF) (Huang et al. 2008) via thiol chemistry as described in 1998 by Storhoff et al. (1998). The AuNPs are visualized using the reflecting mode of a confocal microscope. Gold NPs are well suited for in vivo applications, since they have been shown to be less cytotoxic compared to organic dyes. Next to diagnostic applications, aptamer-modified AuNPs have been used as multimodal-targeting platform, combining diagnostic and therapy (Jaiswal et al. 2002; Michalet 2005).

Fluorescence imaging has been used in vivo even though the tissue-penetrating depth is fairly limited. Deep tissue penetration is only achievable using infrared light. Magnetic resonance imaging (MRI) is free of this limitation. It allows three-dimensional imaging of the whole human body. Nowadays, MRI is employed to differentiate between regular tissue and a solid tumor; however, MRI gains no molecular information. Smart MRI contrast agents, equipped with a targeting unit, are required to achieve this goal. Cell-specific aptamers can be employed to supply specificity to MRI contrast agents, such as iron oxide NPs. In 2007, Smith et al. functionalized iron oxide NPs with either an aptamer towards Burkitt's lymphoma, CCRF-CCM, or Toledo cells. These NPs were shown to magnetically extract the desired target cells from a cell mixture (Smith et al. 2007). Nair et al. developed the so-called nanosurgeon by functionalizing magnetic NPs with the aptamer GB-10, which targets tenascin-C on glioma cells. The nanosurgeons were shown to differentiate between glioma and normal cells. After its binding to the cells, a three-dimensional rotating magnetic field was applied, resulting in surgical action and, thus, in removal of the glioma cells (Nair et al. 2010). The major advantage of the nanosurgeon is based in its ability to differentiate between tumor and healthy cells, resulting in the selective removal of the tumor cells without damaging the surrounding healthy tissue.

10.2.3 On-Chip-Based Approaches

As mentioned previously, the number of circulating cancer cells is low in cancer patients who are in the early state of disease (Oita et al. 2010). Thus, new techniques

need to be developed for efficient capturing and enriching of cancer cells and their subsequent sensitive detection.

On-chip assays have to fulfill two essential prerequisites to be applicable in routine clinical diagnostics: they need to have a high capture efficiency combined with excellent purity of the enriched cell populations. On-chip assays have three major advantages compared to other biosensor systems. First, on-chip assays offer a favored ratio of aptamer to the solution under investigation. A bigger volume of solution can be analyzed while requiring fewer detection reagents. Second, the resident time for cell separation is short. Third, due to the nature of aptamers, on-chip aptamer assays can be efficiently recycled what may reduce costs significantly; aptamers are much more stable to heat, pH, and organic solvents than antibodies and can be denatured and renatured multiple times without significant loss of activity. Phillips et al. (2009) were able to enrich cancer cells in a microfluidic channel modified with sgc8 aptamer to capture CCRF-CEM cells. About 80 % of the cells of interest were captured (capture efficiency) and the purity was 97 % (Phillips et al. 2009). However, since the throughput is too low and the detection not yet optimal, the assay is not applicable for patient samples (Phillips et al. 2009). In a follow-up paper, Xu et al. demonstrated that a microfluidic device subdivided into three regions, each region modified with one type of aptamer (TD05, sgd5, sgc8) specific to one of three leukemia cell lines (Ramos, Toledo, CCRF-CEM), is able to differentiate between the cancer cell lines (Xu et al. 2009). In 2009, Dharmasiri et al. developed a microfluidic device, with PSMA aptamer as a ligand, to isolate and enumerate LNCaP cells (which were used as a model for rare circulating cancer cells) from whole blood. The captured cells were released by trypsination. Both recovery (90 %) and purity (100 %) of cell populations were agreeable with subsequent applications (Dharmasiri et al. 2009). However, despite promising in vitro results, none of the microfluidic devices were tested with clinical samples yet.

Next to enriching cancer cells, it is highly desired to recover viable and physiologically intact cells, which enables cellular analysis by a pathologist. Circulating cancer cells were found to be more susceptive to mechanical stress than cancer cell lines (Liu et al. 2011). Therefore, detaching captured circulating cancer cells using a nondestructive method is necessary for diagnosis in clinical practices (Wan et al. 2011). Cell-specific aptamers, which show temperature-dependent binding, can release their captured targets upon temperature change (Bunka et al. 2010; Sullenger and Gilboa 2002). In this way, an anti-epidermal growth factor receptor (EGFR) aptamer was selected by Ellington et al. in 2010 (Li et al. 2010) and applied for cancer cell isolation followed by microscopic cytology (Wan et al. 2010). Since EGFR is strongly upregulated in a large variety of cancers, this provides a broadly applicable setting to analyze EGFR-dependent cancer cells (Singh and Harris 2005).

Even though a variety of the aptamer-based cell diagnostics are actually designed for clinical applications, none of them is yet used in clinical diagnostics. Aptamers are not limited to detection, but they can furthermore be employed in theragnostic, an approach combining diagnostic and therapy.
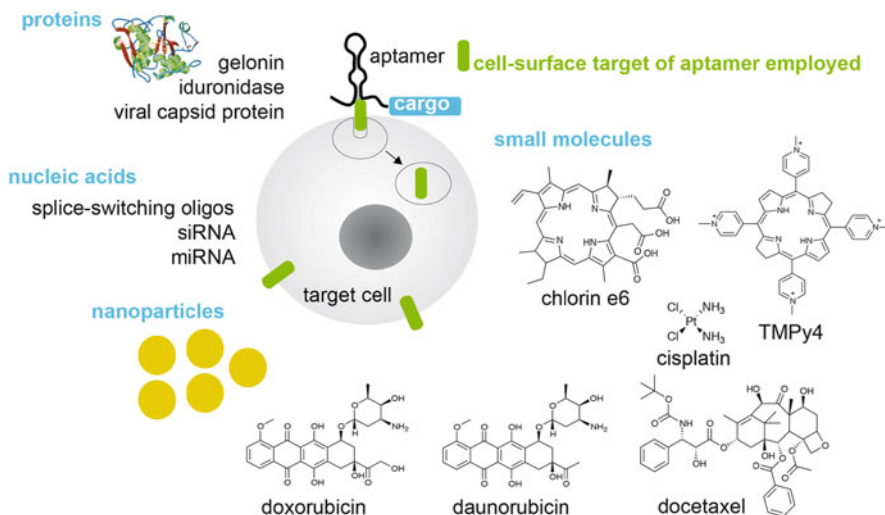
Fig. 10.3 Aptamer-mediated cell—import of various cargo molecules: cell-specific aptamers that are internalized into cells upon target recognition were used as delivery tools for the directed import of various polar molecules. Aptamers have been modified with cargos by covalent bonding, hybridization, or physical conjugation. A variety of cargos like proteins, functional nucleic acids, small molecules, and molecules for photodynamic therapies were successfully delivered via aptamers in a cell-specific manner. Furthermore, aptamer-functionalized nanoparticles were employed as platforms for directed import of multiple molecules. For references see text

10.3   Aptamers as Transport Vehicles

The therapeutic applications of highly polar molecules, such as siRNA, are limited, what is mainly due to their inefficient uptake by cells. A variety of lipids, peptides, and proteins have yet been examined as potential delivery vehicles to overcome this limitation and to facilitate the translocation of polar molecules into cells. For example, siRNAs have been covalently modified with cholesterol (Wolfrum et al. 2007), transferrin (Cardoso et al. 2007), and antibodies (Song et al. 2005) or non-covalently assembled with delivery vehicles, such as folate-conjugated phage RNAs (Guo et al. 2006b). However, most of these targeting molecules lack cellular specificity, and some of them are expensive and time consuming in respect of synthesis. Hence, there is a great demand on delivery molecules combining high specificity and straightforward chemical synthesis. Aptamers combine most of these requirements and consequently have been employed as potential delivery molecules in the recent years. Several groups succeeded in identifying cell-specific aptamers, thereby targeting a plethora of cell-surface receptors. Some of these aptamers have been shown to be actively internalized upon binding to their respective receptor on the cell surface. Therefore, aptamers are highly suitable for the transport of a variety of cargos into specific target cells (Fig. 10.3).

10.3.1 The PSMA Recognizing Aptamer

The most-established and best-characterized aptamers for delivery purposes are the prostate-specific membrane-antigen (PSMA) aptamers A9 and A10, identified by Lupold et al. in 2002 (Lupold et al. 2002). Overexpression of PSMA is correlated with prostate cancer and, thus, is of importance as a diagnostic marker. This integral membrane glycoprotein becomes internalized by endocytosis and, hence, can be used as a protein target for delivery purposes. Farokhzad et al. provided the first report of an aptamer-targeted delivery in 2004, using aptamer-decorated nanoparticles (Farokhzad et al. 2004). They synthesized a functionalized nanoparticle composed of a release polymer coupled to an amine-modified PSMA aptamer (A10) via a carbodiimide coupling on the surface of nanoparticles. As a model drug they encapsulated rhodamine-labeled dextran within those nanoparticles. These nanoparticle–aptamer conjugates significantly are taken up by PSMA-expressing prostate cancer epithelial LNCaP cells. Inversely, they did not enter PSMA-negative PC3 cells. In 2006, this system was taken a significant step further by the same group (Farokhzad et al. 2006). This time, A10-modified nanoparticles loaded with the chemotherapeutic docetaxel (Dtxl) were employed, resulting in a significantly enhanced in vitro cellular toxicity as compared to nontargeted nanoparticles lacking the targeting PSMA aptamer. Furthermore, injection of Dtxl-loaded nanoparticle–aptamer conjugates in subcutaneous prostate cancer xenograft mice resulted in a reduction of the tumor size in vivo. The following years have seen the PSMA aptamer to be continuously used for the cellular delivery of other molecules, such as proteins and siRNA molecules. Chu et al. (2006b) reported the successful delivery of gelonin, a small N-glycosidase protein, which causes cell death by disrupting protein synthesis through cleaving a specific glyco-sidic bond in rRNA. Aptamer–gelonin conjugates not only showed specific inter-nalization but also decreased toxicity of gelonin. In 2006, two groups independently published the cell-specific import of siRNA molecules using PSMA aptamers. Levy and colleagues modularly assembled siRNA–aptamer–streptavidin complexes via biotin chemistry. The ternary complexes were incubated with prostate cancer cells resulting in a siRNA-mediated inhibition of gene expression of lamina A/C or GAPDH. The efficiency of this aptamer-based import was comparable with the use of conventional lipid-based transfection reagents (Chu et al. 2006c). However, immunogenicity may limit the use of streptavidin as a delivery molecule. There-fore, Giangrande et al. developed an approach to covalently append the PSMA aptamer A10 with the sense strand of a siRNA molecule specific for Bcl-2 mRNA (McNamara et al. 2006). Consequently, a chimeric molecule could be generated, which bears a targeting (aptamer) as well as a silencing (siRNA) moiety. The resulting aptamer–siRNA chimera specifically targets prostate cancer cells (LNCaP) and, additionally, acts as a substrate for Dicer resulting in cell-type-specific silencing of antiapoptotic genes, both in vitro and in vivo. Addition of a two-nucleotide overhang at the $3'$ end or swap of the guide and passenger strands results in an optimized chimera with improved silencing and therapeutic efficacy

(Dassie et al. 2009). Wullner et al. (2008) designed a bivalent aptamer-eukaryotic elongation factor 2 (EEF2) targeting siRNA chimera. The design of a chimera with two anti-PSMA aptamers resulted in an increased cellular uptake of siRNAs and an induced EEF2-siRNA-mediated cytotoxicity to prostate cancer cells. During the last years, the interest in aptamer-directed delivery of nanoparticles increased extremely. In 2007, a quantum dot–aptamer–doxorubicin (Dox) conjugate was reported (Bagalkot et al. 2007). The anthracycline intercalates into the double-stranded stem of the aptamer A10, resulting in changes of its fluorescent behavior. Based on this evidence, a donor acceptor system for fluorescence resonance energy transfer (FRET) was developed to sense the release and import of Dox into PSMA-positive prostate tumor cells. The fluorescence of the nanoparticle is quenched when Dox was intercalated, but upon Dox release, the fluorescence of the nanoparticle can be restored (Bagalkot et al. 2007). Farokhazad et al. went a step further and engineered a multifunctional nanoparticle–aptamer conjugate. In this version, the intercalated Dox-aptamer assembly was coupled to docetaxel (Dtxl) nanoparticles. This approach enables the co-delivery of two drugs at the same time (Zhang et al. 2007). In a similar approach, Zhang and coworkers enabled cellular delivery of Dox with A10-modified superparamagnetic iron oxide nanoparticles, allowing treatment and prostate cancer imaging simultaneously (Wang et al. 2008). In 2010, Kim et al. constructed multicomponent nanoplatforms for the co-delivery of small hairpin RNA (shRNA) targeting the antiapoptotic Bcl-2 mRNA combined with Dox (Kim et al. 2010). Therefore, polyplexes were designed that consist of the PSMA aptamer, intercalated Dox, and shRNA. This nanoplatform efficiently and selectively delivered shRNA and the anticancer drug doxorubicin into LNCaP cells. Similarly, an engineered self-assembled nanoparticle allowing the co-delivery of cisplatin and Dtxl to prostate cancer cells was described by Farokhazad and coworkers, which revealed synergistic cytotoxic effects (Kolishetti et al. 2010). However, the use of aptamers for site-specific delivery of anthracyclines in vivo was so far not reported (Fig. 10.4).

10.3.2 Targeting HIV-Infected Cells: The Case of gp120-Binding Aptamers

In 2002, James and coworkers reported the selection of a specific 2′-F-modified RNA aptamer targeting the glycoprotein 120 (gp120) of HIV strain HXB2 (Sayer et al. 2002). This protein plays an important role for the entry of HIV-1 into host cells, whereas it interacts with CD4 and supports the HIV infection by mediating the fusion of the viral with the host cell membrane. Besides Zhou et al. described different aptamers recognizing the HIV-1 glycoprotein of the R5 strain (Khati et al. 2003). Besides their high affinity, these aptamers were also able to inhibit HIV-1 infection of human peripheral blood mononuclear cells (Khati et al. 2003). A few years later, aptamer-siRNA chimeras were generated (Zhou et al. 2009),
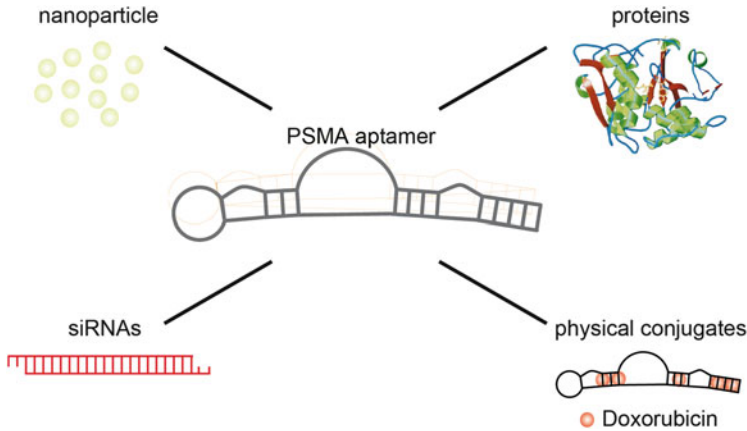
Fig. 10.4 The PSMA aptamer as delivery tool: an RNA aptamer against the prostate membrane antigen (PSMA) serves as delivery vehicle for the import of polar molecules such as doxorubicin (Bagalkot et al. 2006). The PSMA receptor gets internalized via endocytosis, transporting the bound aptamer gelonin (Chu et al. 2006b) complex into the cells (figure modified from Hosur et al. 1995), siRNA (McNamara et al. 2006) against a variety of target mRNAs, or nanoparticles (Farokhzad et al. 2004) to prostate cancer cells

employing gp120 aptamers and HIV-1-specific siRNA molecules. In this approach, aptamer–siRNA chimeras were produced from corresponding double-stranded DNA templates by in vitro transcription, and the functional siRNA duplex was obtained through hybridization with a cognate RNA strand. The anti-gp120-siRNA chimera was not only effective in inhibiting HIV infection of host cells but also Dicer-processed the siRNA domain. Therefore, this chimera represents a dual function molecule in which the aptamer and siRNA portion have potent anti-HIV activities. The group of Rossi selected different aptamers targeting the gp120 protein (Zhou et al. 2009). Based on two of these, they created a series of anti-gp120 aptamer–siRNA chimeras with dual inhibitory function through introduction of a sticky sequence to the aptamer. These sticky sequences were used to modularly conjugate various siRNA molecules (e.g., HIV-1 tat/rev, CD4, and transportin 3-targeting siRNA). The use of Chinese hamster ovary (CHO) cells stably expressing the precursor protein of gp120 (gp160) revealed that these conjugates were internalized into HIV-gp160-positive cells. The siRNA portion was processed by Dicer what results in inhibition of HIV-1 replication and, thus, reducing the infectivity of T cells and primary blood mononuclear cells (PBMCs). Antiviral activity of the gp120 aptamer–siRNA chimera was further analyzed in humanized mouse models. The treatment with either the anti-gp120 aptamer or the aptamer–siRNA, respectively, suppressed HIV-1 replication, whereas the aptamer–siRNA chimera shows a better inhibition and a significantly longer antiviral effect (Neff et al. 2011). To further investigate the adaptability of the anti-gp120 aptamers for the import of various siRNA molecules, Zhou et al. (2011) designed chimeric RNA nanoparticles containing the aptamer coupled to the

packing RNA (pRNA) of the bacteriophage phi29 DNA-packaging motor. These pRNA molecules are able to form dimers, trimers, or hexamers through interaction of interlocking loops. The fusion of the aptamer with pRNA does not affect the ability of the aptamers to bind to its target and, more importantly, inhibit viral replication.

10.3.3   Nucleolin-Targeting G-Quadruplexes

Unlike other cell-specific aptamers, AS1411 was not evolved using SELEX but discovered by testing the possibility of triplex-forming oligodeoxynucleotides to modulate expression of specific genes (Choi et al. 2009). AS1411 is a quadruplex-forming DNA aptamer that recognizes nucleolin and, thus, promotes internalization into cancer cells through hyperstimulation of macropinocytosis (Reyes-Reyes et al. 2010). Once taken up into the cell, AS1411 has been shown to cause a reduction of tumor growth in vitro and in vivo (Ireson and Kelland 2006). As anticancer agent, AS1411 was in clinical trials for the treatment of acute myeloid leukemia (Choi et al. 2009). The target protein of AS1411 is nucleolin, a bcl-2 mRNA-binding protein involved in cell proliferation. The group of Fernandas (Soundararajan et al. 2008) was able to show that AS1411 inhibits the stabilization of the antiapoptotic bcl-2 mRNA and thereby promotes bcl-2 mRNA degradation, which in turn leads to apoptosis. Additionally, AS1411 was reported to alter the localization of the protein arginine methyltransferase 5 (Teng et al. 2007) and to inhibit the activation of nuclear factor-kappaB (Girvan et al. 2006). In 2009, Cao et al. developed an AS1411 aptamer–liposome conjugate, which was able to deliver the chemotherapy drug cisplatin to nucleolin-expressing cancer cells (Cao et al. 2009).

Photodynamic therapy approaches were also realized with AS1411 (Shieh et al. 2010). For that, AS1411 was conjugated with porphyrin-derived molecules, as used during photodynamic therapy regimens, and treatment of MCF-7 breast cancer cells with these conjugates lead to an increased accumulation when compared to nontargeted control cells. Beyond this, nanoparticles were also functionalized with AS1411. As such, the group of Kumar used PLGA–lecithin–PEG nanoparticles coated with AS1411 for the import of the mitotic inhibitor paclitaxel into cancer cells (Aravind et al. 2012). Kim et al. (2012) developed a nanoparticle platform functionalized with AS1411 for the delivery of a microRNA221-molecular beacon, whereas very recently Sullenger et al. described the delivery of so-called splice-switching oligonucleotides to the nucleus of cancer cells (Kotula et al. 2012). They show that these assemblies were delivered to the nucleus and therefore modulate pre-mRNA splicing. Advantageously, AS1411 was not trapped by the endosomal pathway (Kotula et al. 2012). These studies illustrate that AS1411 has been proven to be a useful tool for the delivery of a variety of molecules to tumor cells.

10.3.4 MUC1-Targeting Aptamer

Mucins are glycoproteins expressed by various epithelial cell types protecting the cell surface physicochemically from adverse conditions (Kufe 2009). Additionally, some membrane-associated mucins serve as cell-surface receptors for ligands like lectins, adhesion molecules, or bacteria. Cancer cells revealed differential glyco-sylation and overexpression of mucins (Hollingsworth and Swanson 2004). These mucins are postulated to protect cancer cells from adverse growth conditions and to control the local molecular microenvironment during invasion and metastasis (Hollingsworth and Swanson 2004). For example, the hydrophobic environment created by increased glycosylation inhibits the ability of some chemotherapeutics to access cancer cells. Furthermore, the interaction of mucins with tumor suppressor proteins, such as p53 (Wei et al. 2005) or Akt (Raina et al. 2004), results in inhibition of apoptosis. Beyond the use as tumor marker, the glycoprotein MUC1 becomes internalized as a result of a recycling processes. Ferreira et al. selected aptamers that bind to MUC1, and in a follow-up study, the aptamers were employed for the specific delivery of chlorin e6 (Ferreira et al. 2009). Chlorin e6 is a natural product of the algae chlorella and used as a photosensitizer during photodynamic therapy. Upon irradiation, an increased toxicity due to produced singlet oxygen species of MUC1-positive cancer cells was observed, when treated with aptamer–chlorin e6 conjugates. Minko and coworkers developed a quantum dot system using the same aptamer for the directed import of Dox into cancer cells, where Dox was coupled to the aptamer via a hydrazone bond (Savla et al. 2011). This arrangement enabled the release of Dox inside the target cells and lead to a higher toxicity when compared with free Dox.

10.3.5 Epidermal Growth Factor Receptor-Targeting Aptamer

The EGF receptor has been shown to be involved in many types of cancer (Singh and Harris 2005). After activation and receptor dimerization, it becomes internalized in a clathrin-dependent manner (Madshus and Stang 2009). Ellington and coworkers selected the RNA aptamer J18, which binds EGFR with high affinity (Li et al. 2010). To prove its delivery properties, the aptamer was coupled to gold nanoparticles. Therefore, a complementary capture oligonucleotide covalently attached on the gold surface was used to attach the aptamer onto the nanoparticle. Using flow cytometry, the aptamer J18 was found to specifically and quantitatively direct the delivery of gold nanoparticles to EGFR-expressing cells. Another aptamer, namely, E07, targeting EGFR was reported in 2011 (Li et al. 2011). This aptamer competes with EGF binding and also inhibits cell proliferation of epidermal carcinoma cells (A431). The human epidermal growth factor receptor 2 (HER-2) was also used as a target for the selection of RNA aptamers. Giangrande

et al. developed a specific SELEX approach for the generation of internalized cell-specific aptamers (Thiel et al. 2012). Consequently, the resultant aptamers were able to deliver siRNA, which targets the antiapoptotic gene Bcl-2 into HER-2-positive cells. In this way, aptamer–siRNA-mediated bcl-2 silencing resensitizes the cells to cisplatin.

10.3.6   *Transferrin Receptor Aptamer*

As a cell membrane-associated glycoprotein, the transferrin receptor is involved in cellular uptake of iron and the regulation of cell growth (Daniels et al. 2006). This provides potential for the transferrin-mediated delivery of anticancer drugs into cells that express the transferrin receptors (Kratz et al. 1998). However, the production of transferrin conjugates is a laborious task. Therefore, alternative targeting molecules are needed that recognize the transferrin receptor and which can be easily modified. Consequently, RNA and DNA aptamers targeting the extracellular domain of the mouse transferrin receptor were identified (Chen et al. 2008), and in a proof-of-concept study, the uptake into fibroblasts of the aptamer was shown. In further experiments, the aptamer was conjugated to a lysosomal enzyme (Iduronidase) (Chen et al. 2008). In lysosomal storage diseases, the lack of lysosomal enzymes leads to an accumulation of their respective substrates. Consequently, the delivery of functional lysosomal enzymes with specific aptamers leads to a reduction of substrates in the lysosome and, thus, provides a basis for developing novel treatments (Winchester et al. 2000). Very recently, Levy et al. selected 2′ fluoro-modified RNA aptamers against the human transferrin receptor (CD71), which were readily internalized by human cells (Wilner et al. 2012). In order to assess the potential of the aptamers for delivery approaches, they generate aptamer-functionalized stable nucleic acid lipid particles (Wilner et al. 2012).

10.4   Outlook

Aptamers recognizing cells represent very promising tools for biomedical research. The results and studies described in this book chapter illustrate that cell targeting in general and with aptamers in particular is an emerging field. Aptamers thereby unify chemical and biomedical access and application. The ease by which aptamers can be adapted to various regimens is certainly a strong advantage compared to other targeting ligands, such as antibodies. Chemical modifications of aptamers are nowadays widely used, employed to enhance stability and pharmacological profiles and to facilitate detection and monitoring of aptamers in vivo and in vitro. These results promise a broad future for aptamer-based applications, which however will be only met when robust processes become available for the routine generation of

aptamers even without the in-depth knowledge of an aptamer expert. We believe that due to their sophisticated properties, aptamers will finally make their way into diagnostic and therapeutic areas.

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