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Review

SELEX—A (r)evolutionary method to generate high-affinity nucleic acid ligands

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Abstract

SELEX stands for systematic evolution of ligands by exponential enrichment. This method, described primarily in 1990 [Ellington, A.D., Szostak, J.W., 1990. In vitro selection of RNA molecules that bind specific ligands. Nature 346, 818–822; Tuerk, C., Gold, L., 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 249, 505–510] aims at the development of aptamers, which are oligonucleotides (RNA or ssDNA) binding to their target with high selectivity and sensitivity because of their three-dimensional shape. Aptamers are all new ligands with a high affinity for considerably differing molecules ranging from large targets as proteins over peptides, complex molecules to drugs and organic small molecules or even metal ions. Aptamers are widely used, including medical and pharmaceutical basic research, drug development, diagnosis, and therapy. Analytical and separation tools bearing aptamers as molecular recognition and binding elements are another big field of application. Moreover, aptamers are used for the investigation of binding phenomena in proteomics. The SELEX method was modified over the years in different ways to become more efficient and less time consuming, to reach higher affinities of the aptamers selected and for automation of the process. This review is focused on the development of aptamers by use of SELEX and gives an overview about technologies, advantages, limitations, and applications of aptamers.

Keywords: SELEX; Aptamer; Random oligonucleotide library; Target

Contents

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1. Introduction

Since its first description in 1990, the SELEX technology is widely applied as an in vitro selection method to evolve nucleic acid ligands, called aptamers, with new functionalities. The term aptamer is derived from the Latin word ''aptus''—which means fitting (Ellington and Szostak, 1990) and the Greek word ''meros'' meaning particle. Aptamers are short single-stranded nucleic acid oligomers (ssDNA or RNA) with a specific and complex three-dimensional shape characterized by stems, loops, bulges, hairpins, pseudoknots, triplexes, or quadruplexes. Based on their three-dimensional structures, aptamers can well-fittingly bind to a wide variety of targets from single molecules to complex target mixtures or whole organisms (Fig. 1). Binding of the aptamer to the target results from structure compatibility, stacking of aromatic rings, electrostatic and van der Waals interactions, and hydrogen bondings, or from a combination of these effects (Hermann and Patel, 2000). The first aptamers developed consisted of unmodified RNA (Ellington and Szostak, 1990; Tuerk and Gold, 1990). Later on, single-stranded DNA aptamers for different targets were described (Ellington and Szostak, 1992) as well as aptamers containing chemically modified nucleotides (Green et al., 1995). Chemical modifications can introduce new features into the aptamers, improve their binding capabilities or enhance their stability (Gold et al., 1995). Numerous variants of the original SELEX process were described to select aptamers with high affinities and specificities for their targets. Many of the selected aptamers show affinities comparable to those observed for monoclonal antibodies. In addition, aptamers can distinguish between chiral molecules and are able to recognize a distinct epitope of a target molecule (Michaud et al., 2003; Jenison et al., 1994). Thus, the differentiation between closely related targets (e.g. theophylline and caffeine) is possible. A further revolutionary aspect of the SELEX technology is the selection of ligands beyond natural systems by use of chemically produced oligonucleotide libraries, without the constraints imposed by having to be selected or produced in a living organism. The big variety of 10^{15} different oligonucleotides within the SELEX library and the amplification steps of target-binding oligonucleotides during the selection process facilitate far higher possibilities to select ligands with highest affinity than natural selection. Moreover, the in vitro selection process for aptamers can be carried out under conditions akin to those used in the assay for which the aptamer is being developed—the aptamer will maintain its structure and function in the final assay and will not dissociate, which can be a problem with antibodies (Mukhopadhyay, 2005). The SELEX conditions can be further modified to direct the selection to aptamers with desired features. This stands in contrast to the classical production of antibodies, where it is not possible to influence such parameters and therefore leaving it limited to physiological conditions (Jayasena, 1999).

This review will give an overview of the SELEX technology. The different steps of a SELEX process are discussed in detail with regard to the selection of target-binding aptamers. Advantages and limitations, as well as the versatile application potential of aptamers are described. The in vitro selection of nucleic acids with catalytic activity, like ribozymes and DNAzymes are not included in this review.

2. SELEX technology

2.1. General principle

Combinatorial chemistry is an important technology for industry as well as biotechnological and pharmaceutical research to discover new materials or molecules with desirable properties, new drugs, and catalysts. It is characterized by the synthesis and simultaneous screening of large libraries of related, but structurally distinct compounds to identify and isolate functional molecules. Nucleic acids are very attractive compounds for combinatorial chemistry, because they are able to fold into defined secondary and tertiary structures, and they can be amplified by PCR or in vitro transcription easily. Very complex libraries of random sequence oligonucleotides with about 10^{15} different molecules can be produced by chemical synthesis and screened in parallel for a particular functionality, such as high-affinity ligand-binding (aptamers) or catalytic activity (ribozymes, DNAzymes). In 1990, a new method was described, using a combinatorial nucleic acid library to select RNA oligonucleotides (aptamers) that bind very tightly and selectively to a certain non-nucleic acid target. Tuerk and Gold studied the interaction between the bacteriophage T4 DNA polymerase (gp43) and the ribosome-binding site of the mRNA, which encodes the enzyme. They selected gp43 binding sequences from an RNA pool randomized at specific positions, and called this selection procedure SELEX—Systematic Evolution of Ligands by Exponential enrichment (Tuerk and Gold, 1990). Ellington and Szostak independently used a similar selection procedure to isolate – from a random sequence RNA library – RNA molecules, with the ability to fold into a stable three-dimensional structure, thus creating a specific binding site for small ligands, e.g. Cibacron Blue and Reactive Blue 4 (small organic dyes). They named these selected, individual RNA sequences 'aptamers' (Ellington and Szostak,

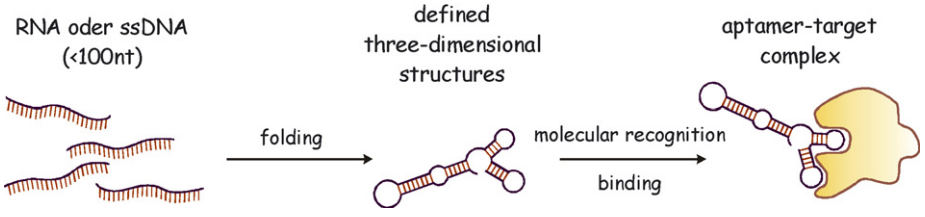

Fig. 1. Schematic representation of the functionality of aptamers.

1990). Two years later, the successful selection of single-stranded DNA sequences from a chemically synthesized pool of random sequence DNA molecules could be shown (Ellington and Szostak, 1992). These DNA aptamers were able to recognize and bind the ligands (Cibacron Blue and Reactive Green 19) in the same way like RNA aptamers. Since this early phase of the SELEX technology it became an important and widely used tool in molecular biological, pharmaceutical, and medical research. Additionally, this technique often was modified to select aptamers for different applications.

Basic steps of a SELEX process are presented in Fig. 2. Iterative cycles of in vitro selection and enzymatic amplification mimic a darwinian type process driving the selection towards relatively few, but optimized structural motifs as solution for a given problem (e.g. ligand binding) (Göringer et al., 2003). Starting point of a typical SELEX process is a chemically synthesized random DNA oligonucleotide library consisting of about 10^{13} to 10^{15} different sequence motifs (James, 2000). In a SELEX procedure which is directed to the selection of DNA aptamers, this library can be used without any pretreatments, whereas a conversion into an RNA library has to accomplish prior starting an RNA SELEX process. For more details concerning the initial random oligonucleotide library see Section 2.3. In either case the randomized RNA or DNA pool is incubated directly with the target. The binding complexes are subsequently partitioned from unbound and weakly bound oligonucleotides. This is one of the most crucial aspects of an aptamer selection process and strongly affects binding features of the aptamers to be selected (see Section 2.4). Target bound oligonucleotides are eluted and amplified by PCR (DNA SELEX) or reverse transcription (RT)-PCR (RNA SELEX). The resulting double-stranded DNA has to be transformed into a new oligonucleotide pool by separating the relevant ssDNA or by in vitro transcription and subsequent purifying the synthesized RNA. This new and enriched pool of selected oligonucleotides is used for a binding reaction with the target in the next SELEX round. By iterative cycles of selection and amplification the initial random oligonucleotide pool is reduced to relatively few sequence motifs with the highest affinity and specificity for the target. The number of rounds

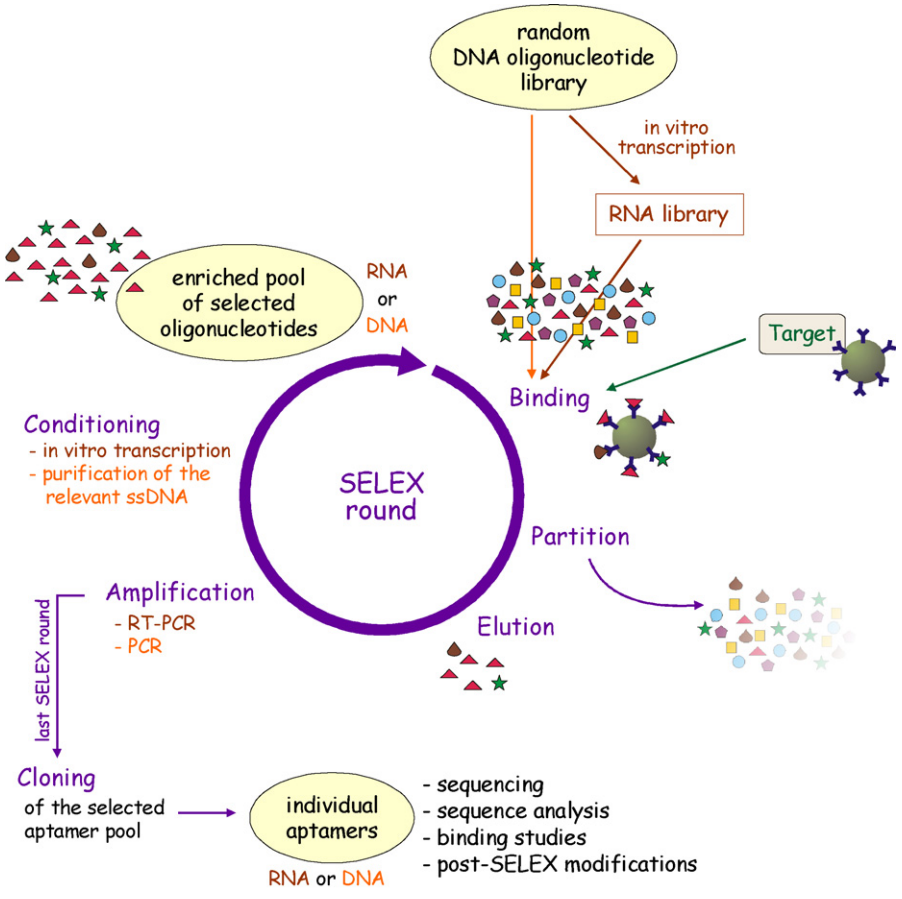

Fig. 2. In vitro selection of target-specific aptamers using SELEX technology. Starting point of each SELEX process is a synthetic random DNA oligonucleotide library consisting of a multitude of ssDNA fragments with different sequences ($\sim 10^{15}$). This library is used directly for the selection of DNA aptamers. For the selection of RNA aptamers the library has to be transferred into an RNA library. The SELEX procedure is characterized by the repetition of successive steps consisting of selection (binding, partition, and elution), amplification and conditioning. In the first SELEX round the library and the target molecules are incubated for binding. Unbound oligonucleotides are removed by several stringent washing steps of the binding complexes. The target-bound oligonucleotides are eluted and subsequently amplified by PCR or RT-PCR. A new enriched pool of selected oligonucleotides is generated by preparation of the relevant ssDNA from the PCR products (DNA SELEX) or by in vitro transcription (RNA SELEX). This selected oligonucleotide pool is then used for the next selection round. In general, 6 to 20 SELEX rounds are needed for the selection of highly affine, target-specific aptamers. The last SELEX round is finished after the amplification step. The enriched aptamer pool is cloned and several individual aptamers have to be characterized.

necessary depends on a variety of parameters, such as target features and concentration, design of the starting random DNA oligonucleotide library, selection conditions, ratio of target molecules to oligonucleotides, or the efficiency of the partitioning method. Additional steps can be introduced into each round of the SELEX process particularly with regard to the specificity of the oligonucleotides. Negative selection steps or subtraction steps are strongly recommended to minimize an enrichment of unspecifically binding oligonucleotides or to direct the selection to a specific epitope of the target. The affinity of the oligonucleotides to their target can be influenced by the stringency of the selection conditions. Typically, the stringency is progressively increased in the course of a SELEX process. This can be achieved by reducing the target concentration in later SELEX rounds or changing the binding and washing conditions (buffer composition, volume, time) (Marshall and Ellington, 2000).

The detection of an enrichment of target-specific oligonucleotides indicates that the SELEX process is finished. The last SELEX round is stopped after the amplification step and the PCR products are cloned to get individual aptamer clones from the selected pool. These individual aptamers are sequenced and sequence analyzed. Representative aptamer clones are chosen and used in binding assays to characterize their binding features in more detail including the affinities and specificities. Mutation and truncation experiments can be performed to narrow down the minimal binding region within the aptamer sequence. Finally, most of the selected aptamers are subjected to some post-SELEX modifications, e.g. with the view to enhance the stability of the aptamers (incorporation of modified nucleotides), in order to use the aptamers in analytical detection assays or for target purification (attachment of reporter groups, functional groups or linker molecules) (see Section 3).

The SELEX process for the selection of target-specific aptamers is a universal process characterized by repetition of the five mentioned main steps (binding, partition, elution, amplification, and conditioning); however there is no standardized aptamer selection protocol for any target. The SELEX design and the specific selection conditions depend, e.g. on the target itself, the oligonucleotide library or the desired features and application of the aptamers to be selected. The following subchapters give more detailed information about targets, libraries and the particular process steps.

2.2. Target molecules

Since 1990, the SELEX technology has been applied to different classes of targets. Inorganic and small organic molecules, peptides, proteins, carbohydrates, antibiotics, as well as complex targets like target mixtures or whole cells and organisms were used for an aptamer selection. Several publications summarized the various target molecules according to certain aspects (Famulok, 1999; Wilson and Szostak, 1999; Göringer et al., 2003; Klussmann, 2006). Examples of aptamer targets are shown in Table 1.

Aptamers can be developed for molecules connected with nucleic acids (nucleotides, cofactors) and for nucleic acid binding proteins like enzymes (polymerases) or regulatory proteins, but also for molecules by nature not associated with nucleic acids like growth factors (Green et al., 1996; Jellinek et al., 1994) or organic dyes (Ellington and Szostak, 1990, 1992). Beside organic molecules, divalent metal ions were also used as target in SELEX experiments. Ciesiolka et al. and Hofmann et al. described the selection of RNA aptamers with affinities for Zn^{2+} and Ni^{2+}, respectively. In both cases an affinity matrix charged with those metal ions was used for the isolation of selective binding RNA molecules from a randomized RNA library, adopting a method for the purification of proteins with an extension of histidine. The smallest molecular target so far used for an aptamer selection is ethanolamine, a simple structured molecule (C_2 chain) with two functional groups (–OH, $–NH_2$) (Mann et al., 2005). In few examples the SELEX technology was also applied to select aptamers targeting nucleic acid structures. Tertiary RNA structures play an important role in several biological processes, e.g. as regulatory domains of gene expression. Aptamers could help to understand such structures. By interfering biological processes mediated by tertiary RNA structures, e.g. in pathogens, these aptamers could also function as therapeutic oligonucleotides (Duconge and Toulme, 1999; Toulme et al., 2003). According to the ratio among the publications concerning aptamers, most of the aptamers were selected for proteins or for peptides as special epitopes of a

Table 1
Examples of targets used for aptamer selections

Target for aptamer selection	Type of aptamer	K_D	References
Inorganic components			
Zn^{2+}	RNA	1.2 mmol/L	Ciesiolka et al. (1995)
Ni^{2+}	RNA	0.8–29 μmol/L	Hofmann et al. (1997)
Small organic molecules			
Ethanolamine	DNA	6–19 nmol/L	Mann et al. (2005)
Theophylline	RNA	100 nmol/L	Jenison et al. (1994)
Malachite green	RNA	1 μmol/L	Grate and Wilson (2001)
Organic dyes	RNA	100–600 μmol/L	Ellington and Szostak (1990)
	DNA	33–46 μmol/L	Ellington and Szostak (1992)
Sulforhodamine B	DNA	190 nmol/L	Wilson and Szostak (1998)
Hematoporphyrin	DNA	1.6 μmol/L	Okazawa et al. (2000)

Table 1 (*Continued*)

Target for aptamer selection	Type of aptamer	K_D	References
Ricin toxin	DNA	58–105 nmol/L	Tang et al. (2006)
Cholic acid	DNA	5–67.5 μmol/L	Kato et al. (2000)
4,4′-Methylenedianiline	RNA	0.45–15 μmol/L	Brockstedt et al. (2004)
Dopamine	RNA	2.8 μmol/L	Mannironi et al. (1997)
Cocaine	DNA	n.s.[a]	Stojanovic et al. (2000)
Nucleotides and derivatives			
Adenine	RNA	10 μmol/L	Meli et al. (2002)
ATP (adenosine)	RNA	0.7–50 μmol/L	Sassanfar and Szostak (1993)
Adenosine/ATP	DNA	6 μmol/L	Huizenga and Szostak (1995)
ATP	RNA	4.8–11 μmol/L	Sazani et al. (2004)
Xanthine	RNA	3.3 μmol/L	Kiga et al. (1998)
cAMP	RNA	10 μmol/L	Koizumi and Breaker (2000)
Cofactors			
Coenzyme A	RNA	n.s.[a]	Saran et al. (2003)
Cyanocobalamin	RNA	88 nmol/L	Lorsch and Szostak (1994)
Riboflavin	RNA	1–5 μmol/L	Lauhon and Szostak (1995)
FMN	RNA	0.5 μmol/L	Burgstaller and Famulok (1994)
FAD	RNA	137–273 μmol/L	Burgstaller and Famulok (1994)
NAD	RNA	n.s.[a]	Burgstaller and Famulok (1994)
	RNA	2.5 μmol/L	Lauhon and Szostak (1995)
S-adenosyl methionine	RNA	n.s.[a]	Burke and Gold (1997)
S-adenosyl homocysteine	RNA	0.1 μmol/L	Gebhardt et al. (2000)
Biotin	RNA	5 μmol/L	Wilson et al. (1998) and Wilson and Szostak (1995)
Nucleic acids			
TAR RNA element of HIV-1	DNA	50 nmol/L	Boiziau et al. (1999)
	RNA	20–50 nmol/L	Duconge and Toulme (1999)
	DNA	50 nmol/L	Sekkal et al. (2002)
Yeast phenylalanine tRNA	RNA	12–26 nmol/L	Scarabino et al. (1999)
E.coli 5S RNA	RNA	6–12 μmol/L	Ko et al. (1999)
	RNA	3 μmol/L	Ko et al. (2001)
Amino acids			
L-Arginine	RNA	330 nmol/L	Geiger et al. (1996)
	DNA	~2.5 mmol/L	Harada and Frankel (1995)
L-Citrulline	RNA	62–68 μmol/L	Famulok (1994)
L-Valine	RNA	12 mmol/L	Majerfeld and Yarus (1994)
L-Isoleucine	RNA	1–7 mmol/L	Lozupone et al. (2003)
	RNA	200–500 μmol/L	Majerfeld and Yarus (1998)
D-Tryptophan	RNA	18 μmol/L	Famulok and Szostak (1992)
L-tyrosinamide	DNA	45 μmol/L	Vianini et al. (2001)
L-histidine	RNA	8–54 μmol/L	Majerfeld et al. (2005)
Carbohydrates			
Cellobiose	DNA	600–nmol/L	Yang et al. (1998)
Sialyl Lewis X	RNA	0.085–10 nmol/L	Jeong et al. (2001)
Chitin	DNA	n.s.[a]	Fukusaki et al. (2000)
Sialyllactose	DNA	4.9 μmol/L	Masud et al. (2004)
Sephadex	DNA	n.s.[a]	Srisawat et al. (2001)
Antibiotics			
Kanamycin A	RNA	≤300 nmol/L	Lato et al. (1995)
Kanamycin B	RNA	180 nmol/L	Kwon et al. (2001)
Streptomycin	RNA	n.s.[a]	Wallace and Schroeder (1998)
Neomycin	RNA	~100 nmol/L	Wallis et al. (1995)
Tobramycin	RNA	2–3 nmol/L	Wang and Rando (1995)
Lividomycin	RNA	≤300 nmol/L	Lato et al. (1995) and Lato and Ellington (1996)
Moenomycin A	RNA	300–400 nmol/L	Schürer et al. (2001)
Tetracycline	RNA	1 μmol/L	Berens et al. (2001)
Chloramphenicol	RNA	25–65 μmol/L	Burke et al. (1997)
Peptides and proteins			
T4 DNA polymerase	RNA	5–30 nmol/L	Tuerk and Gold (1990)

Table 1 (*Continued*)

Target for aptamer selection	Type of aptamer	K_D	References
α-Thrombin	DNA	200 nmol/L	Bock et al. (1992)
	RNA	<1–4 nmol/L	White et al. (2001)
Bovine thrombin	RNA	164–240 nmol/L	Liu et al. (2003)
Neurotensin receptor NTS-1 (rat)	RNA	0.37 nmol/L	Daniels et al. (2002)
Immunglobulin E	DNA	23–39 nmol/L	Mendonsa and Bowser (2004)
	RNA	30–35 nmol/L	Wiegand et al. (1996)
	DNA	10 nmol/L	Wiegand et al. (1996)
Interferon-γ	RNA	2.7 nmol/L	Kubik et al. (1997)
MCP-1 (mouse)	RNA	180–370 pmol/L	Rhodes et al. (2001)
PDGF	DNA	0.1 nmol/L	Green et al. (1996)
VEGF	RNA	0.1–2 nmol/L	Jellinek et al. (1994)
	RNA	0.05–0.13 nmol/L	Ruckman et al. (1998)
HIV-1 integrase	RNA	10–800 nmol/L	Allen et al. (1995)
HIV-1 RT	RNA	∼5 nmol/L	Tuerk et al. (1992)
	DNA	∼1 nmol/L	Schneider et al. (1995)
	DNA	180–500 pmol/L	Mosing et al. (2005)
HIV-1 nucleocapsid protein	RNA	0.84–1.4 nmol/L	Kim et al. (2002)
TTF1	DNA	3.3–67 nmol/L	Murphy et al. (2003)
HGF	DNA	19–25 nmol/L	Saito and Tomida (2005)
Streptavidin	RNA	7 nmol/L	Tahiri-Alaoui et al. (2002)
	RNA	70–200 nmol/L	Srisawat and Engelke (2001)
	DNA	57–85 nmol/L	Stoltenburg et al. (2005)
L-Selectin	DNA	1.8–5.5 nmol/L	Hicke et al. (1996)
Taq DNA polymerase	DNA	0.04–9 nmol/L	Dang and Jayasena (1996)
Prion protein (PrP^c)	RNA	0.1–1.7 nmol/L	Proske et al. (2002)
PrP^{Sc} fibrils	RNA	23.4 nmol/L	Rhie et al. (2003)
$rPrP^c$	RNA	n.s.[a]	Weiss et al. (1997)
$rPrP^c$	DNA	n.s.[a]	Takemura et al. (2006)
C5 protein	RNA	2–5 nmol/L	Biesecker et al. (1999)
Hepatitis C virus RdRp	DNA	1.3/23.5 nmol/L	Jones et al. (2006)
Hepatitis C virus NS3	RNA	n.s.[a]	Kumar et al. (1997)
Hepatitis C virus NS3 helicase	DNA	140 nmol/L	Zhan et al. (2005)
ppERK2/ERK2	RNA	4.7/50 nmol/L	Seiwert et al. (2000)
Protein kinase C delta	DNA	122 nmol/L	Mallikaratchy et al. (2006)
RNase H1	DNA	10–80 nmol/L	Pileur et al. (2003)
Colicin E3	RNA	2–14 nmol/L	Hirao et al. (2004)
Oncostatin M	RNA	7 nmol/L	Rhodes et al. (2000)
Tumour marker MUC1	DNA	0.1–34 nmol/L	Ferreira et al. (2006)
GnRH	L-RNA	190 nmol/L	Leva et al. (2002)
	L-DNA	45 nmol/L	
Vasopressin	L-DNA	1.2 μmol/L	Williams et al. (1997)
Amyloid peptide βA4(1-40)	RNA	29–48 nmol/L	Ylera et al. (2002)
Microcystin	DNA	1 mmol/L	Nakamura et al. (2001)
Complex structures			
Anthrax spores	DNA	n.s.[a]	Bruno and Kiel (1999)
Ribosomes/ribosomal protein S1	RNA	4-5 nmol/L	Ringquist et al. (1995)
Rous sarcoma virus (RSV)	RNA	n.s.[a]	Pan et al. (1995)
Differentiated PC12 cells	DNA	n.s.[a]	Wang et al. (2003)
Transformed YPEN-1 endothelial cells/pigpen	DNA	n.s.[a]	Blank et al. (2001)
U251 glioblastoma cells/tenascin-C	DNA	150 nmol/L	Daniels et al. (2003)
	RNA	5 nmol/L	Hicke et al. (2001)
Live African trypanosomes	RNA	60 nmol/L	Homann and Göringer (1999)
Jurkat T cell leukemia	RNA	n.s.[a]	Lee and Lee (2006)
Receptors of host-cell matrix molecules on Trypanosoma cruzi	RNA	40–400 nmol/L	Ulrich et al., 2002
Leukemia cells CCRF-CEM	DNA	0.8–229 nmol/L	Shangguan et al. (2006)

[a] n.s., not specified.

protein of interest. Proteins exhibit very large, multifunctional surfaces, which make them excellent aptamer targets. Thrombin was the first protein target used for an aptamer selection that normally does not interact with nucleic acids (Bock et al., 1992). The anti-thrombin DNA aptamer folds into a G-quartet structure and is able to inhibit thrombin function. This aptamer is one of the best studied aptamers and is widely used as model system for aptamer applications. In addition to defined single target molecules the SELEX technology can also be applied to complex target mixtures, whole cells, tissues, and organisms. In this case the final aptamer pool can be more complex dependent on the number and abundance of potential target molecules, but also on the affinity of the aptamers. In most of these SELEX experiments directed towards complex mixtures the selected aptamers are targeting cell surface molecules, often proteins. Aptamer selection can be for example directed at alterations in the cell surface structures caused by changes of the environmental conditions or by diseases (Blank et al., 2001; Ulrich et al., 2002; Wang et al., 2003; Göringer et al., 2003).

The multitude of different targets used in SELEX experiments implicates that the selection of aptamers is possible for virtually any target. However, there are some general prerequisites for a potential target to successfully select aptamers with high affinity and specificity. Defined single target molecules should be present in sufficient amount and with high purity. This helps to minimize the enrichment of unspecifically binding oligonucleotides and to increase the specificity of the selection. Some target features that facilitate an aptamer selection are positively charged groups (e.g. primary amino groups), the presence of hydrogen bond donors and acceptors and planarity (aromatic compounds) (Wilson and Szostak, 1999; Rimmele, 2003). The aptamer selection is more difficult for targets with largely hydrophobic character and for negatively charged molecules (e.g. containing phosphate groups). These target requirements are caused by the basic principles of the intermolecular interactions in an aptamer–target complex. The aptamers bind to their targets by a combination of complementarity in shape, stacking interactions between aromatic compounds and the nucleobases of the aptamers, electrostatic interactions between charged groups or hydrogen bondings (Patel, 1997; Patel et al., 1997; Hermann and Patel, 2000). In presence of the target, and on formation of the binding complex, the aptamers undergo adaptive conformational changes. The folding into defined three-dimensional structures permits the aptamers to completely encapsulate small target molecules by generating a specific binding pocket. In higher molecular weight targets like proteins, different substructures on the molecule surfaces are involved in aptamer binding. For example, side chains of basic amino acids (lysine, arginine) are often responsible for intermolecular hydrogen bondings.

The binding complexes of aptamers targeting nucleic acids are mostly characterized by loop–loop interactions (kissing complexes) between two hairpin structures or involving internal loops and bulges. Triple-stranded complexes can also be generated by binding of a single-stranded domain or oligonucleotide to a double-stranded nucleic acid (Klussmann, 2006).

2.3. Starting random DNA oligonucleotide library

Starting point of a SELEX process is a chemically synthesized random DNA oligonucleotide library. This library consists of a multitude of ssDNA fragments ($\sim 10^{15}$ molecules) comprising a central random region of 20–80 nt flanked by different specific sequences of 18–21 nt, which function as primer binding sites in the PCR (Fig. 3). For the selection of DNA aptamers, this library can be used directly in the first round of a DNA SELEX process. Sense and antisense primer derived from the specific sequences at the 5′-end and 3′-end enable the amplification of the selected oligonucleotides in each SELEX round. Some researchers prefer a large scale amplification of the random DNA library before initiating the aptamer selection process in order to eliminate damaged DNA synthesis products, which cannot be amplified by PCR (Marshall and Ellington, 2000). Moreover, it is assumed that chemically synthesized DNA molecules are amplified by the polymerase with different efficiency. Therefore, it is possible to lose some of the target-binding sequences after the first selection round. An initial PCR step also provides several copies of the original oligonucleotides that can serve as amplification templates.

For the selection of RNA aptamers, the random DNA oligonucleotide library has to be transformed into a RNA library before starting the first round of an RNA SELEX process. A special sense primer with an extension at the 5′-end containing the T7 promoter sequence and an antisense primer are necessary to convert the ssDNA library into a dsDNA library by PCR. The dsDNA is then in vitro transcribed by the T7 RNA polymerase resulting in a randomized RNA library, which is used to start an RNA SELEX. During this SELEX process the selected RNA of each round has to be reverse transcribed and subsequently amplified by RT-PCR using the same primer as described above. The new RNA pool for the next SELEX round is then generated again by in vitro transcription.

Important aspects for designing the oligonucleotide library are the size of the randomized region, the type of randomization and chemical modifications of the DNA or RNA. Oligonucleotide libraries with randomized regions of 20–80 nt are commonly used for in vitro selection experiments. A great number of selected aptamers are truncated down to a minimal functional sequence after the SELEX process. For example, Bock et al. (1992) selected an aptamer for the human thrombin from a DNA library of 96-mer oligonucleotides comprising a 60 nt random sequence. They found that the functionality of a truncated aptamer variant containing only the 15-mer consensus sequence was retained. This suggests that libraries with short randomized regions are sufficient for a successful aptamer selection. Short libraries are better manageable, cost-effective in chemical synthesis and the selection of rather short aptamers is preferred for many applications. However, longer randomized regions give the libraries a greater structural complexity. This is important particularly for targets which are not known to be associated with

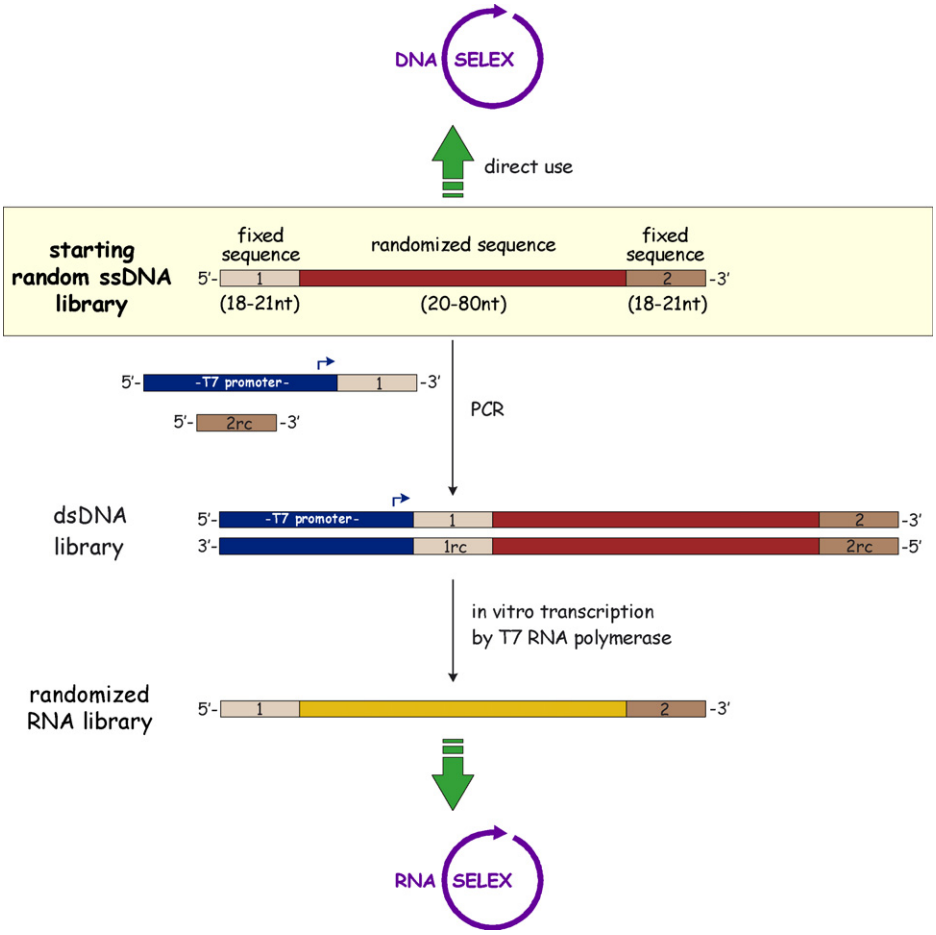

Fig. 3. Starting random DNA oligonucleotide library. The classical SELEX process for the selection of target-specific aptamers starts with a chemically synthesized DNA oligonucleotide library. Each oligonucleotide is characterized by an internal randomized sequence of 20–80 nt flanked by two fixed sequences (termed 1 and 2), which act as primer binding sites in the PCR. This library is used directly for the selection of DNA aptamers (DNA SELEX). For the selection of RNA aptamers (RNA SELEX) the library has to be converted to a dsDNA pool by PCR followed by in vitro transcription to generate a randomized RNA library. Two special primers are needed for the PCR. The antisense primer (termed 2rc) is reverse complementary to the fixed sequence at the 3′-end of the library. The sense primer is derived from the fixed sequence at the 5′-end and is modified by an extension containing the T7 promoter sequence (termed-T7 promoter-/1). The T7 RNA polymerase specifically recognizes this promoter and permits an in vitro transcription to synthesize the RNA library for starting an RNA SELEX.

or to bind to nucleic acids. Therefore, a longer random sequence pool may provide better opportunities for the identification of aptamers (Marshall and Ellington, 2000). In addition, the possibility for an interaction between the oligonucleotides and the targets over an extended domain of both binding partners during the SELEX process seems to contribute to the selection of high-affinity aptamers (Klussmann, 2006).

The majority of the SELEX experiments utilize an oligonucleotide library with a completely randomized intern region. Davis and Szostak (2002) designed a partially structured RNA library to increase the chance of selecting functional nucleic acid structures, in this case GTP-specific aptamers. This library was characterized by a stable stem-tetraloop structure (12 nt) located in the center of a 52 nt random region. The stem-loop should act as a structural anchor for the formation of specific recognition loops. The analysis of the selected GTP-binding aptamers suggested that this partially structured library was a superior source of high-affinity aptamers. Nutiu and Li (2005) designed a partially randomized DNA library with the goal to directly select structure-switching aptamers. This library contained a specific sequence of 15 nt flanked by two random sequences of 10 nt and 20 nt, which were further flanked by primer-binding sequences. The specific sequence within the random region was complementary to a biotinylated capture oligonucleotide and thus the library could be immobilized on an avidin coated surface by DNA hybridization. Oligonucleotides which are able to bind to the target should be released from the surface as a result of switching their structures for formation of the binding complexes. This SELEX strategy is very attractive for the selection of aptamers targeting small molecules, because an immobilization of the target is not necessary. In addition, the selected aptamers can easily be transformed into signaling aptamers, e.g. by modification of the capture oligonucleotide with a quencher molecule in combination with a fluorophore bearing antisense oligonucleotide that also hybridizes to the aptamer sequence. In presence of the target the capture oligonucleotide is released due to the switching of aptamers structure and the fluorescence intensity increases significantly by dequenching.

Besides natural nucleic acids, chemically modified oligonucleotide libraries were used in some SELEX experiments with the goal to increase the complexity of a library, to introduce new features like functional groups providing new possibilities for the interaction with target molecules, to enhance the stability of oligonucleotide conformations, or to increase the resistance to nucleases, which is important for many applications of aptamers (Jayasena, 1999; Klussmann, 2006; Kopylov and Spiridonova, 2000; Kusser, 2000). There are a great number of new functionally modified nucleotides to build up such oligonucleotide libraries for the aptamer selection, but a crucial aspect is their compatibility to enzymes used in the SELEX process. Typical modifications concern the 2′-position of the sugar in RNA libraries. The ribose 2′-OH group of pyrimidine nucleotides is replaced with a 2′-NH_2 or 2′-F group, which protects the RNA from degradation by nucleases. Furthermore, RNA libraries containing 2′-O-methyl substituted nucleotides were also described for aptamer selection (Burmeister et al., 2005). New functionalities can be incorporated in nucleic acids by modifications of the nucleobases commonly at the C-5 position of pyrimidines or at the C-8 position of purines (Kopylov and Spiridonova, 2000). Kuwahara et al. (2006) demonstrate the incorporation of a variety of chemically modified nucleotides into DNA by KOD Dash DNA polymerase during PCR. A series of C-5-modified dUTPs bearing amino acids were used to prepare a variety of modified DNAs. The authors mentioned that such modified oligonucleotides with new functionalities are applicable to in vitro selection experiments particularly to select DNA aptamers for anionic target molecules. For example C-5-amino substituted pyrimidines were used in oligonucleotide libraries for SELEX experiments to select cationic-charged modified aptamers targeting sialyllactose and ATP, respectively (Masud et al., 2004; Vaish et al., 2003). 5-Bromouracil and 5-iodouracil were used to generate photo-cross-linkable aptamers, which can be activated by UV irradiation to form a covalent linkage with the bound target (Golden et al., 2000).

Modifications of the phosphate backbone of nucleic acids were also applied to in vitro selections of aptamers. A common modification is the replacement of the non-binding oxygen in the phosphodiester linkage by sulfur (Andreola et al., 2000). This produces a phosphorothioate linkage, and thus increases the resistance of phosphorothioate oligonucleotides against nuclease digestion. A successful selection of so called ‘thioaptamers’ by phosphorothioate SELEX was demonstrated by Jhaveri et al. (1998) and King et al. (2002).

Some other modifications of oligonucleotide libraries serve for the quantification of selected oligonucleotides during the SELEX process. In general, radioactive labeled nucleotides are incorporated or fluorescent molecules are attached to the 5′-end of the oligonucleotides.

2.4. Selection

The selection step of the SELEX procedure includes the binding of the target molecules with the oligonucleotide library, the subsequent partition of unbound oligonucleotides, and final elution of bound oligonucleotides. The selection is designed to find out those molecules from the big variety of the oligonucleotide library with the greatest affinity and specificity for the target of interest. Therefore, the oligonucleotide library is exposed directly to the target and allowed to incubate for a period of time. The direct interaction of the oligonucleotides with the target during consecutive binding steps of the SELEX process is closely application-oriented. An in-depth knowledge about the respective target is not necessary for the aptamer selection, which is a big advantage.

One of the crucial steps of a SELEX process with outstanding importance for the selection of aptamers with high affinity and specificity is the efficient partitioning between target-binding and non-binding oligonucleotides. The immobilization of the target molecule on a particular matrix material allows an effective separation. In this case, the oligonucleotide library is incubated for binding with the immobilized target. The use of affinity chromatography with immobilization of target on column material like sepharose or agarose is a conventional method for this separation step (Liu and Stormo, 2005; Tombelli et al., 2005a). However, substantial amounts of target are necessary to obtain a high efficient loading of the column. The use of magnetic beads offers another possibility for target immobilization (Stoltenburg et al., 2005; Kikuchi et al., 2003; Lupold et al., 2002; Murphy et al., 2003). This method requires only very small amounts of target and enables a very simple handling. Moreover, the magnetic beads allow parallel procedures to some degree, ‘‘by hand’’ as well as automated.

A commonly used method of separation without target immobilization consists in ultrafiltration by use of nitrocellulose filters with distinct molecular weight cut-offs (Bianchini et al., 2001; Schneider et al., 1993; Tuerk and Gold, 1990). Thereby, losses of target-binding oligonucleotides as well as unspecific interactions of oligonucleotides with the membrane resulting in their non-specific enrichment are possible but undesired effects. During the last years, several authors described further possible methods of separation during the SELEX process, e.g. Capillary Electrophoresis, CE (Mendonsa and Bowser, 2004; Mosing et al., 2005; Tang et al., 2006), Flow Cytometry, FC (Davis et al., 1997; Yang et al., 2003), Electrophoretic Mobility Shift Assay, EMSA (Tsai and Reed, 1998), Surface Plasmon Resonance, SPR (Misono and Kumar, 2005) or centrifugation (Rhie et al., 2003; Homann and Göringer, 1999). An alternative approach (but only when the target is a protein) is to allow the target molecule and the aptamer to mix freely in solution before recovering the aptamer–protein complex on a cyanogens bromide (CNBr) activated sepharose or StrataCleane™ Resin (Stratagene Inc.). The protein is selectively adsorbed and therefore any aptamer attached to it, whereas free aptamers are not (Sampson, 2003). In some cases the target protein is tagged with special molecules for better capturing of the aptamer–target complex by the column material (Kim et al., 2002; Kimoto et al., 2002). Gopinath (2007) gives a very good overview of partition methods used in SELEX procedures.

For assessment of the SELEX procedure, quantification of the enriched target-binding oligonucleotides as well as the amount of non-binding oligonucleotides of each selection round have to be determined. Mostly radioactive markers are used for the quantification during a SELEX process (Beinoraviciute-Kellner et al., 2005; Ellington and Szostak, 1990; Shi et al., 2002). This is a very sensitive method enabling the detection of slightest amounts of nucleic acids. A drawback of the technology is the necessity to manage the whole process in an isotope laboratory, which is very cost-intensive. Moreover, the use of radioactive material is not environmentally compatible and holds a health risk for employees. Alternatively, fluorescence labels may be used for quantification (Stoltenburg et al., 2005; Davis et al., 1997; Rhie et al., 2003). By this means, operation in a molecular biological standard laboratory is possible, additional costs are avoided and a satisfactory high sensitivity for quantification and detection of nucleic acids is gained.

After discarding the unbound molecules in most cases the binding oligonucleotides are eluted of the target for following steps in the SELEX process. But there are also examples in literature where aptamer–target complexes are subsequently processed unseparated (Bruno, 1997; Missailidis et al., 2005). Methods for aptamer elution could be denaturing methods like heat treatment (Stoltenburg et al., 2005) or addition of special substances as urea, SDS or EDTA (Bianchini et al., 2001; Theis et al., 2004; Weiss et al., 1997). Other researchers carry out affinity elution by use of the target (Famulok, 1994; Geiger et al., 1996) or elution with competitive binders (Bridonneau et al., 1999). Cho et al. developed a photoelution method by application of a photocleavable linker (Cho et al., 2004b; Chung et al., 2005).

A big advantage of the SELEX procedure is the possibility to adapt the conditions of further applications already during the selection process. On account of this, numberless variations of the procedure originally established by Tuerk and Gold (1990) were described during the last years. Some of these methods were developed to increase affinity or specificity of the selecting aptamers, others to optimize the procedure. Table 2 gives an overview of SELEX variants described in literature.

2.5. Amplification

Because of the high complexity of the initial oligonucleotide library it is normally expected to get only few functional oligonucleotides in result of the selection step. Therefore, these very rare active molecules are amplified. Beside the purpose of enrichment of the selected aptamer pool, it is also possible to attach modifications via special primers during the amplification step. On this way it is possible to attach additional

Table 2
Examples of SELEX procedure modifications

Designation	Description	References
Negative SELEX	- Minimizes the co-selection of unwanted nucleic acid ligands (e.g. for immobilization matrix) - Preselection with molecules, which should not be recognized, removing of unwanted oligonucleotide structures from the pool	Blank et al. (2001), Haller and Sarnow (1997), Vater et al. (2003) and Geiger et al. (1996)
Counter-SELEX or subtractive SELEX	- Generates aptamers which are able to discriminate between closely related structures - Introducing of a selection step to the related target for elimination of aptamers from the oligonucleotide pool, which are not able to distinguish between the related structures	Jenison et al. (1994), Geiger et al. (1996) Haller and Sarnow (1997), Lee and Lee (2006), Shangguan et al. (2006), Wang et al. (2003) and White et al. (2003)
Blended SELEX	- To give additional properties to aptamers beyond the binding capability - Enlarging of nucleic acid molecules by special non-nucleic acid components	Smith et al. (1995) and Radrizzani et al. (1999)
Expressions cassette SELEX or SELEX-SAGE	- Special forms of Blended SELEX (with transcription factors) - Optimizes aptamer activity for gene therapy applications	Martell et al. (2002) and Roulet et al. (2002)
Chimeric SELEX	- Using of two or more different libraries for production of chimerical aptamers with more than one wanted feature or function - Each of the parent libraries will be selected first to a distinct feature, then fusion of the selected aptamers	Burke and Willis (1998)
Multi-stage-SELEX	- Special form of Chimeric SELEX - After fusion of preselected aptamer components, reselection to the entirety of the targets	Wu and Curran (1999)
Deconvolution-SELEX	- To generate aptamers for complex targets - Discrimination between relevant aptamers (binding to distinct target structures within the complex mixture) and irrelevant oligonucleotides	Blank et al. (2001) and Morris et al. (1998)

Table 2 (*Continued*)

Designation	Description	References
Covalent SELEX or cross-linking SELEX	- Aptamers containing reactive groups which are capable of covalent linking to a target protein	Jensen et al. (1995) and Kopylov and Spiridonova (2000)
Photo-SELEX	- Aptamers bearing photo-reactive groups - Bind and photo cross-link to a target and/or photo activate a target molecule	Brody et al. (1999), Golden et al. (2000), Jensen et al. (1995) and Johnson and Gershon (1999)
SPIEGELMER®-Technology	- Selection with ordinary D-nucleic acids for the mirrored (enantiomer) target - Synthesis of the resulting aptamers as L-isomers which bind now to the originally un-mirrored target	Eulberg and Klussmann (2003), Klussmann et al. (1996) and Faulhammer et al. (2004)
Tailored-SELEX	- Integrated method to identify aptamers with only 10 fixed nucleotides through ligation and removal of primer binding sites within the SELEX process - Useful for selection of short aptamers and Spiegelmers	Vater et al. (2003)
Signaling aptamers or molecular beacons	- Aptamers which report target binding by switching their structures and show a signal (e.g. fluorescent)	Jhaveri et al. (2000) and Rajendran and Ellington (2003)
Genomic SELEX or cDNA-SELEX	- Construction of a SELEX library of an organism's genome (e.g. cDNA fragments) - Target proteins or metabolites from the same organism are used to identify meaningful interactions - Allows the identification of protein targets directly from mRNA pools	Dobbelstein and Shenk (1995), Singer et al. (1997), Gold et al. (1997a,b), Zolotukhin et al. (2001), Chen et al. (2003), Shimada et al. (2005) and Wen and Gray (2004)
Toggle-SELEX	- Switching (''toggling'') between targets during alternating rounds of selection	White et al. (2001) andBianchini et al. (2001)
Indirect selection	- Selection target (e.g. metal ions) is not the actual binding partner of the aptamer, but binding is target-dependent (occurs only in presence of the target)	Kawakami et al. (2000)
In vivo selection	- One possibility to select RNA-processing signals - Uses transient transfection in an iterative procedure in cultured vertebrate cells	Coulter et al. (1997)
Tissue SELEX	- Method for generating aptamers capable of binding to complex tissue targets such as collections of cells in diseased tissues	Daniels et al. (2003) and Morris et al. (1998)
TECS-SELEX	- Cell-surface displayed recombinant or natural protein is directly used as the selection target	Ohuchi et al. (2006) and Shangguan et al. (2006)
FluMag-SELEX	- DNA oligonucleotides with a fluorescein modification - Target immobilization on magnetic beads	Stoltenburg et al. (2005)
CE-SELEX	- Use of capillary electrophoresis (CE) For separation	Mendonsa and Bowser (2004), Mosing et al. (2005), Tang et al. (2006) and Drabovich et al. (2006)
Non-SELEX	- A process that involves repetitive steps of partitioning with no amplification between them - Non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) used for partitioning	Berezovski et al. (2006)
EMSA-SELEX	- Use of electrophoretic mobility shift assay (EMSA) for partitioning in every round	Tsai and Reed (1998)
Use of nanoManipulator-atomic Force Microscope (nM-AFM)	- Only one round of selection necessary - Picking up and visualizing of singled aptamer-target-complexes by nM-AFM - Affinity measurement of single aptamer-target-complexes possible by dynamic force spectroscopy - Amplification by single-molecule-PCR	Guthold et al. (2002)
On-chip selection	- Selection (in combination with a method for point mutations) and analyzing of DNA aptamers on chips	Asai et al. (2004)
Yeast genetic selection	- In vivo optimization of in vitro-preselected aptamers - Library with degenerated aptamers - Use of a yeast three(one)-hybrid system	Cassiday and Maher (2003)

functional groups for detection, immobilization, enlargement, etc., to DNA aptamers.

At this stage, SELEX processes for the generation of RNA and DNA aptamers differ significantly. RNA oligonucleotides firstly have to be passed through a reverse transcription PCR (RT-PCR). As a result the corresponding cDNA is achieved, which is amplified in a subsequent PCR. One of the applied primers has to bear a T7 promoter for RNA polymerase at the 5′-end. In contrast, ssDNA aptamers merely have to be amplified by PCR, where special primers can be used to provide the aptamers with additional properties.

2.6. Conditioning

The conditioning step is necessary to prepare the amplified oligonucleotide pool for the next cycle of the SELEX process. After the preceding PCR, the enriched pool is available as dsDNA. A transcription with T7 RNA polymerase has to follow in case of RNA aptamers. The resulting RNA molecules are used as input in the following SELEX round. In case of ssDNA aptamers, single strand separation has to be carried out. Several methods are described in literature for this purpose. Many researchers use the streptavidin/biotin system but in different ways. Some add the biotin molecule to the unwanted strand and use the arising size difference in gel electrophoresis to distinguish between both strands (Fitzwater and Polisky, 1996). Others let the dsDNA (only one strand biotinylated) bind to streptavidin surfaces (beads or plates) and separate the strands after DNA denaturation (Naimuddin et al., 2007). Another possibility is to perform an asymmetric PCR which uses only one or a much bigger amount of one primer to obtain ssDNA products (Wu and Curran, 1999). Two methods which are very easy to handle are shown in Fig. 4. Both methods create a size difference between the strands by use of modified primers. For instance, a 3′-terminal ribose residue (riboU) PCR primer is used in the method of Walder et al. (1993). The second method works with a primer that bears a hexaethyleneglycol (HEGL) spacer and a 20 adenine nucleotide lengthener (Williams and Bartel, 1995). The resulting size difference becomes visible in a subsequent denaturing polyacrylamide gel electrophoresis by UV shadowing (Hendry and Hannan, 1996) or by fluorescence if fluorophore modified primer were used in PCR (Stoltenburg et al., 2005).

2.7. Cloning and characterization

By iteratively executing the procedures of selection, amplification and conditioning during the SELEX process, the complexity of the original library is reduced and target-binding candidates are enriched. As soon as the affinity of the enriched library cannot be increased any further – generally after 6–20 selection rounds – usually the nucleotide sequences of individual binding molecules are determined. For this purpose, the final pool is cloned into a bacterial vector and individual colonies are sequenced. The number of different aptamers obtained is a function of both, of the target itself and of the stringency of the selection, and can vary from 1 to 1,000,000 (Conrad et al., 1995). Commonly, about 50 or more aptamer clones are analyzed by sequencing and sequence analysis. Sequence alignments are very useful to assess the complexity of the selected aptamer pool and to identify aptamers with homologous sequences. For performing alignments Internet programs like, e.g. CLUSTAL W (http://www.ebi.ac.uk/clustalw/) are frequently used (Chenna et al., 2003; Thompson et al., 1994). Based on the alignment data, the aptamer clones with mostly identical sequences, different in only few single nucleotide positions, can be grouped. In some cases special sequence patterns or highly conserved regions can be identified among the aptamer groups. These regions are often involved in the specific target binding of the aptamers.

Secondary structure analysis of the aptamer sequences also provides information about relevant structures for binding. Such analyses are usually carried out by the program *mfold*

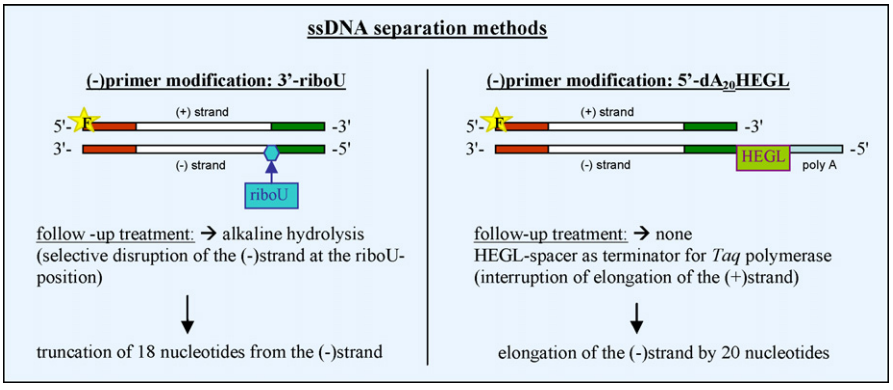

Fig. 4. Two methods for ssDNA separation. The displayed methods are used for separation of the wanted ssDNA, the (+)strand, after PCR. Special primers are applied during PCR. One method (left side) uses a 3′-end riboU modification, which bears a single ribonucleotide containing uracil. During the follow-up alkaline hydrolysis with sodium hydroxide, the unwanted (−)strand disrupts selectively at the riboU-position. The result is a truncation of the (−)strand by some nucleotides. The other method (on the right of the figure) works with a primer that contains a hexaethyleneglycol (HEGL) spacer at its 5′-end and an extension of several adenine nucleotides (polyA). The HEGL-spacer acts as a terminator for *Taq* polymerase. The elongation of the (+)strand stops, while the (−)strand grows further. No follow-up treatment is necessary. In both methods the created size difference can be identified in subsequent electrophoresis by UV shadowing or by fluorescence (use of fluorophore modified primer for the (+)strand). The wanted strand is cutted out and processed further.

which is also available per internet (http://www.bioinfo.rpi.edu/applications/mfold/dna/form1.cgi). This program of Zuker (2003) calculates the possible configuration of single-stranded nucleic acids by energy minimizing method considering stems, loops and bulges. The determined consensus motifs often are located in stem-loop structures (Horn et al., 2004; Jiang et al., 1997; Lee et al., 2005; Nishikawa et al., 2004), but they are even found in different secondary or tertiary structures. For example, several authors describe conspicuous G-rich binding motifs which form G-quadruplexes (Andreola et al., 2001; Macaya et al., 1993; Wilson and Szostak, 1998). Others found pseudoknot formations that are responsible for target binding (Burke et al., 1996; Chaloin et al., 2002; Nix et al., 2000).

Subsequent binding studies for determination of specificity and affinity of the selected aptamers usually follow. Commonly the individual aptamer clones are tested, but it is also possible to utilize the enriched oligonucleotide pool of the last SELEX round. This pool of aptamers consists in a characteristic mixture analog a polyclonal antibody serum (Bruno and Kiel, 1999).

Binding studies are a very important part of aptamer selection. Feasible subsequent applications of aptamers depend on the exact ascertainment of specificity and affinity. In this context, the determination of the affinity constant (dissociation constant K_D), as characteristic parameter of an aptamer, is of major importance. A small K_D-value designates a high affinity to the target. Attention should be taken on the fact that neither specificity nor affinity is regulated by size, complexity, or quantity of the target. These aptamer characteristics are rather determined by target attributes like functional groups and the chosen properties during the SELEX process.

A further point is to define the aptamer–target interaction. Therefore, besides other binding experiments, footprinting studies often are performed (Brown et al., 1997; Cho et al., 2004a; Harada and Frankel, 1995; Tahiri-Alaoui et al., 2002). Furthermore, the three-dimensional binding structure of aptamer and target has to be clarified. In this regard methods like nuclear magnetic resonance (NMR) spectroscopy, X-ray structure analysis, and crystallization are usually applied (Forster et al., 2006; Kelly et al., 1996; Schneider and Suhnel, 1999; Soukup and Breaker, 1999).

3. Post-SELEX modifications

Post-SELEX modifications are executed either in order to increase the stability of the selected aptamers or to optimize binding parameters to the target or relevant molecules. For further applications, modifications with functional groups for detection or immobilization are possible.

In order to enhance biostability of the aptamers, it is possible to introduce – after a successful selection – similar chemical modifications as was the case for initial oligonucleotide libraries above-mentioned in Section 2.3. For instance, a changing of the 2′-OH groups of ribose to 2′-F or 2′-NH_2 groups or 2′-O-methyl substituted nucleotides protects the RNA from degradation by nucleases (Green et al., 1995; Rhodes et al., 2000; Ruckman et al., 1998). A 3′-end capping with streptavidin-biotin, inverted thymidine (3′-idT, creates a 3′-3′ linkage) or several 5′-caps (amine, phosphate, polyethyleneglycol (PEG), cholesterol, fatty acids, proteins, etc.) defends oligonucleotides from exonucleases (Dougan et al., 2000; Klussmann, 2006; Marro et al., 2005).

Locked nucleic acids (LNAs) (Petersen and Wengel, 2003) hold great promise to stabilize aptamers, because of their substantially increased helical thermostability and excellent mismatch discrimination when hybridized with RNA or DNA; furthermore, they are resistant to degradation by nucleases. In LNA nucleotides, post-SELEX modified, the sugar is made bicyclic by covalently bridging the 2′-oxygen and the 4′-carbon with a methylene (Darfeuille et al., 2004; Klussmann, 2006; Schmidt et al., 2004).

As was mentioned above, sequence truncations are performed to narrow down the binding region of the target. Regions that are not important for direct interaction with the target or for folding into the structure that facilitate target binding are removed. Sometimes such truncations even result in raised affinity (Burke et al., 1996; Ruckman et al., 1998; Wilson and Szostak, 1998).

Properties (affinity or specificity) of a selected aptamer or aptamer pool generally can be improved by reselection. The aptamer (aptamer pool) is subjected to a second selection process, in which the existing aptamer sequences are diversified by modification or mutagenesis in order to generate a new library (Held et al., 2003; Huang and Szostak, 2003). Mutagenized oligonucleotides could be prepared by error-prone PCR (Bittker et al., 2002) or random fragmentation/elongation of preselected aptamers (Hwang and Lee, 2002; Stelzl and Nierhaus, 2001). Post-SELEX modified aptamers as well can be subjected to reselection after the modification procedure in order to guarantee binding to desired targets. An example of the success of such a reselection process can be seen in a SELEX using Rous sarcoma virus (RSV) as a target. Initially, an unmodified RNA library was applied. After the successful aptamer selection, a modification of the binding aptamers with 2′-fluoropyrimidines followed which resulted in a total loss of affinity of the aptamers for RSV. However, several rounds of reselection with modified nucleotides resulted in binders that could link to RSV and interfere with viral replication just like the unmodified pool, while increasing the stability of the aptamer (Pan et al., 1995).

Downstream modifications are performed to adapt the selected oligonucleotides for further applications. For instance, aptamers are equipped with functional groups for immobilization (e.g. biotin, amino groups) or detection (e.g. fluorescent molecules). Substitutions with 5-bromouracil and 5-iodouracil are used to create photo-cross-linkable aptamers (Kimoto et al., 2004; Sekiya et al., 2006). Moreover, aptamers could be fused to combine different features. In that way for instance Yoshida et al. (2006) developed an aptameric subunit which was composed of an enzyme-inhibiting aptamer and a target-binding aptamer for biosensing of target molecules.

4. Automated SELEX

In order to develop aptamer sensor arrays that can be used for analyzing molecular mixtures, a fast and parallel devel-

opment of multiple aptamers is desirable. Therefore, considerable efforts aimed at the development of automated platforms for aptamer selection. Automation of the selection procedure means the integration and automation of different molecular biology methods (for binding, partioning, elution, amplification, conditioning) and is thus a very complex automation challenge.

A robotic work station configuration was set up for the selection of nucleic acid ligands. It is based on an augmented Beckmann Biomek 2000 Pipetting robot (Cox et al., 1998) which was adapted to the selection of aptamers for a protein (hen egg white lysozyme) by some modifications, including the substitution of magnetic separation by vacuum-filter separation (Cox and Ellington, 2001). The generated aptamer had an affinity of K_D = 31 nmol/L for the cognate protein and inhibited its enzymatic function. The authors declare that the described robotic work station can carry out eight selections in parallel and will complete approximately 12 rounds of selection in 2 days. Aptamers against 120 targets should be produced in 1 month, which exceeds the rate of throughput by a factor of 10–100 from manual to automated selection. However, the elimination of ''quality control steps'' routinely applied during manual selections, may result in accumulation of artifacts or even the failure of selection (Cox and Ellington, 2001).

Aptamers for some other proteins (CYT-18, Rho, MEK 1) were selected with this robotic work station (Cox et al., 2002b). Moreover, an automatic characterization of aptamers *via* binding reactions and the recording of binding isotherms in order to determine the respective binding constants is integrated into the work station (Cox et al., 2002b). A further extension of the automatic work station consists in the generation of protein targets directly transcribed and translated from the respective gene in vitro on the robotic work station (Cox et al., 2002a). This should further accelerate aptamer selection for proteins and increase the utility of aptamers as reagents in proteome analysis.

As manually operated SELEX-procedures are mostly successful if the progress of enrichment is monitored and the selection conditions are adapted to the monitoring results, an automated system should be designed as flexible as possible. The flexibility mostly concerns the adjustment of stringency or the variation of incubation times as well as the availability of online monitoring of the amplification step. Eulberg et al. (2005) have developed an automated SELEX process with high flexibility and versatility in terms of choice of buffers and reagents as well as stringency of selection conditions. They selected RNA aptamers for the mirror-image confirmation (D-peptide) of substance P. The mirror-image conformation of the truncated aptamer (Spiegelmer) binds the naturally occurring L-substance P with a K_D of 40 nmol/L. The selection robot is based on a RoboAmp 4200 E instrument (MWG Biotech, Ebersberg, Germany) with further modifications for ultrafiltration, fluorescence detection, and semi-quantitative PCR. The described robotic system performs two selection rounds per day and is, therefore, rather slow in comparison to the system described by Cox and Ellington (2001), but it is more flexible and the samples are treated almost exactly as a human researcher would do, increasing the chance of success (Eulberg et al., 2005).

The prototype of a microfluidic, microline-based assembly that uses LabVIEW controlled actuatable valves and a PCR machine was used for the selection and synthesis of an anti-lysozyme aptamer (Hybarger et al., 2006). The anti-lysozyme aptamer generated by the microfluidic prototype and that generated using the established automated robot are identical in sequence. The system is organized in a modular fashion, which enables the modification and evaluation of individual processes. The chip-based microfluidic environment should combine the advantages of miniaturization which have been documented in connection with chip-based enzymatic assays, immunoassays and nucleic acid detection with the selection of aptamers by SELEX (Hybarger et al., 2006). The microfluidic platform for automated SELEX experiments should lead to the development of standardized protocols and even ''SELEX kits'', making automated aptamer selection easier accessible to many investigators. The possibility of high-throughput SELEX would enable producing a multitude of aptamers in a short time, which can be used as ligands for proteomics and metabolomics.

5. Advantages and limitations of aptamers and their selection technology

The basic SELEX technology is a powerful and universal tool to evolve aptamers able to bind very tightly and specifically to a wide variety of targets. Hundreds of publications concern the selection of aptamers and their applications, which reflect the great interest for this research field and its enormous potential for pharmacy and medicine, as well as environmental analytics. The main advantages of aptamers and their selection technology are summarized in the following listing; some of them were already discussed in more detail in the chapters before.

- The SELEX process is applicable to different classes of targets. Besides defined single targets, complex target structures or mixtures without proper knowledge of their composition are suitable for a successful aptamer selection.
- Aptamers can be selected with high affinities and specificities for their targets. The affinities are often comparable to those observed for antibodies. Most of the calculated K_D values are in the low nanomolar to picomolar range dependent on the measuring principle.
- In contrast to antibodies, aptamers are selected by an in vitro process independent of animals or cell lines. Thus, the SELEX process is also applicable under non-physiological conditions.
- The selection of aptamers for toxic target molecules or for molecules with no or low immunogenicity is possible.
- Various modifications can be introduced in the basic SELEX process to direct the selection to desired aptamer features or to intended applications of the aptamers.
- Several methods were developed to modify aptamers after their selection, mostly to enhance their stability or to permit

the quantification and immobilization of the aptamers (e.g. chemical modifications at the nucleotide level, attaching functional groups and reporter molecules, terminal capping). Further optimizations of the selected aptamers are possible with regard to their affinities and specificities (e.g. truncation, reselection).

- Depending on the SELEX conditions, a limited manual parallelization of the process is possible. Furthermore, automated SELEX protocols were described and established as robotic systems.
- Selected aptamers can be produced by chemical synthesis with high accuracy and reproducibility. No variation between different production charges is anticipated (Kawazoe et al., 1996). They are purified by stringent, denaturing conditions ensuring very high purity.
- Denatured aptamers can be regenerated easily within minutes, which is important for many applications.
- Aptamers are nucleic acid species, and thus exhibit a very high shelf-life.
- Aptamers can be delivered into cells or expressed within cells (intramers) by transcription of expression cassettes. Such strategies permit the investigation of the function and interplay of proteins in the context of living cells or organisms (Famulok et al., 2001). Aptamers could be used for intracellular detection of target molecules or could function as intracellular inhibitors (Burgstaller et al., 2002).
- The small size relative to antibodies makes aptamers easier to be synthesized and chemically modified, and enables them to access protein epitopes that might otherwise be blocked or hidden (Lee et al., 2006). Low molecular weight aptamers are important tools for imaging analysis or cytometry, because they can reach targets in the cells (Ulrich et al., 2004).
- Aptamers display low to no immunogenicity, which is important for animal or human therapeutic applications (Nimjee et al., 2005b).
- Using aptamers as therapeutic drugs, matched antidotes can easily be produced, which are able to selectively control the drug activity (Rimmele, 2003; Rusconi et al., 2004).
- The published aptamer works also reveal several limitations of aptamers and their selection technology. Such experiences are valuable for starting new SELEX experiments, because some limitations can be overcome by modifying the SELEX conditions.
- In reality, not any target molecule is suitable for an aptamer selection (see Section 2.2).
- There is no standardized SELEX protocol applicable for any kind of target. The selection conditions have to be adapted to the current circumstances (target, desired features of the aptamers, applications).
- The majority of the published aptamers were manually selected. Thus, the whole process to get high affine and specific aptamers (iterative SELEX rounds, cloning of the aptamer pool, characterization) is time consuming.
- Repeated selection of already known aptamer sequences in different SELEX processes is possible in dependence of target composition and SELEX conditions.
- The enrichment of unspecific binding oligonucleotides during the aptamer selection process is often observed. In order to solve this problem, the SELEX conditions have to be adapted accurately to current requirements, e.g. negative selection steps are recommended.
- Aptamers are relatively unstable in biological fluids. Different strategies were developed to overcome this problem, e.g. chemical modifications can significantly improve the stability.
- They may be prone to non-specific binding in complex (biological) samples, which makes it difficult to quantify the target by use of aptamer based assays, and may necessitate sample pre-treatment (Guthrie et al., 2006).
- High-affinity aptamers are not, until now, as widely available as antibodies (Guthrie et al., 2006).

6. Fields of applications of aptamers

In spite of the very encouraging promises, research on aptamers is still at the beginning. One disadvantage is the unavailability of a standardized protocol for aptamer development which is applicable without specific modifications for different targets. But aptamer research is catching up for faster results. Medical and pharmaceutical basic research as well as clinical diagnostic and therapy (e.g. inhibition of enzyme activities, blocking of receptor binding sites) offer a big field of application (Klug et al., 1999; Hermann and Patel, 2000; Thiel, 2004). Aptamers are starting to compete antibodies as therapeutic agents. Comprehensive overviews about aptamers with therapeutic applications are given in Nimjee et al. (2005b) and Ulrich et al. (2006). In the field of therapy, several aptamers for medically relevant targets will be tested regarding their application potential. The first approved aptamer with therapeutic function is the anti-human VEGF aptamer (VEGF: vascular endothelial grow factor) (Tucker et al., 1999). The PEGylated form of this aptamer was called Pegaptanib and used as the medicinal active component of the newly developed drug for the treatment of wet age related macular degeneration. The pharmaceutical product Macugen® (pegaptanib sodium injection) from Pfizer Inc./OSI Pharmaceuticals was approved in December 2004 (USA) and January 2006 (Europe) (Maberley, 2005; Chapman and Beckey, 2006). Beyond this, the development of aptamers for therapeutics and biomedicine is pushed in broad front (Yan et al., 2005). Some companies are very active within this field. For example, Archemix Inc., USA (http://www.archemix.com), Noxxon Pharma AG, Germany (http://www.noxxon.net), Isis Innovation Ltd., UK (http://www.isis-innovation.com), and SomaLogic Inc., USA (http://www.somalogic.com/) having aptamer based therapeutics under product development or in clinical studies. In addition, the research on aptamers for application in medicine is far advanced for tumor imaging and therapy (e.g. Hicke et al., 2006; Ferreira et al., 2006; White et al., 2003; Herr et al., 2006), influenza virus detection and inhibition (e.g. Jeon et al., 2004; Gopinath et al., 2006) as well as clinical diagnostic in general (e.g. Guthrie et al., 2006) (see some examples in Table 3).

Table 3
Examples of applied aptamers

Target	Aptamer/assay	Field of application	Research or product state	Reference
VEGF	RNA aptamer, chemically modified	Therapy, wet age related macula degeneration	Product: Macugen® (Pfizer Inc.)	Maberley (2005) and Chapman and Beckey (2006)
Thrombin	DNA aptamer (thrombin inhibitor ARC-138)	Therapy, anticoagulant	Product development, Phase 1 studies in August 2004 (Archemix Corp.)	Nimjee et al. (2005a)
Factor IXa	RNA aptamer (factor IXa inhibitor) and its antidote REG1	Therapy, anticoagulant	Product development, Phase 1 studies completed in 2006, (Regado Biosciences Inc.; Archemix Corp.)	Nimjee et al. (2005a) and Dyke et al. (2006)
L-2-Phenoxy-propionic acid	RNA aptamer/L-RNA chiral stationary phase	Capillary electrochromatography, enantiomer separation of acid herbicides	Research	Andre et al. (2006)
Ricin	RNA aptamer	Capillary electrophoresis with laser induced fluorescence detection, detect. limit: 500 pM, assay time < 10 min	Research	Haes et al. (2006), Hesselberth et al. (2000) and Kirby et al. (2004)
Abrin toxin	DNA aptamer/molecular luminescence switch $[Ru(phen)_2(dppz)]^{2+}$	Medicine/safety, detect. limit: 1 nM, lin. range: 1–400 nM	Research	Tang et al. (2007)

The big market of immunological diagnostics in the medical area will create an increasing competition between antibody- and aptamer-based test kits. Some working groups are already starting to transfer antibody based diagnostic platforms to aptamers. The enzyme linked oligonucleotide assay (ELONA), based on ELISA technology, is one example for using aptamers instead of antibodies (Drolet et al., 1996). In all probability, aptamers will be applied in standardized diagnostic test kits in the near future.

Aptamers in general are more suitable than antibodies for escorting and in vivo imaging, reviewed in Hicke and Stephens (2000). Because of their small size, aptamers can penetrate into cells and tissues more effectively than antibodies. But in contrast to antibodies, aptamers have a faster clearance rate, which is advantageous particularly for potentially cytotoxic agents like therapeutic radiolabels. If a longer aptamer circulation in the body is required, aptamers can be modified to extend their bloodstream half-life (Yan et al., 2005).

Besides their use in the pharmaceutical area, aptamers are used for molecular detection in analytical systems (Clark and Remcho, 2002). Examples for the application of aptamers as specific ligands in affinity chromatography (Deng et al., 2001), affinity capillary electrophoresis (German et al., 1998), capillary electrochromatography (Kotia et al., 2000; Connor and McGown, 2006) and flow cytometry (Davis et al., 1998) were described. The application of aptamers for the separation, purification and quantification of analytes in chromatography, electrochromatography and capillary electrophoresis techniques in general is described in detail and with examples by Ravelet et al. (2006). DNA and RNA aptamers have been applied to the separation/purification of proteins and separation of small molecules and enantiomers. In capillary electrophoresis, aptamers are mainly used for the separation of species and the characterization of affinity interactions. Ravelet et al. (2006) state the great potential of these molecular tools in the separation science field. However, the broad practical applications of aptamers in routine analysis are limited by reasons mentioned in Section 5. Moreover, for big separation units, a high amount of aptamers is necessary, causing high costs in comparison to other separation materials. Therefore, the use of aptamers for separation units is limited mainly to miniaturized systems.

In future, aptamers will also play an important role as new receptors in biosensors (O'Sullivan, 2002; Tombelli et al., 2005b). Examples of aptamer applications mostly with optical detection of the binding reaction are described. For example, a thrombin aptamer and an L-adenosin aptamer were used as biosensor receptors (Kleinjung et al., 1998; Lee and Walt, 2000; Potyrailo et al., 1998). Another biosensor development is based on anti-human IgE aptamers and a quartz crystal microbalance for detection (Liss et al., 2002). For a biosensor application it is very important, that target binding and denaturation of aptamers are reversible.

A very sophisticated application of aptamers aims at the development of logic gates (''AND''/''OR'') and related smart materials, where aptamer targets are used as the chemical stimuli for controllable cooperativeness of aptamer-coated nanomaterials (Liu and Lu, 2006).

7. Future perspectives

Aptamers for therapeutic applications promise a good stroke of business among the aptamer applications. In this area we find the furthermost advanced commercialization with Macugen®, a therapeutic against wet age related macula degeneration, produced by Pfizer Inc. This first demonstration of a successful aptamer based drug development will prod other companies like Archemix, SomaLogic, Regado Biosciences, Noxxon, NascaCell, AptaRes and others to follow with their own aptamer based drugs.

Furthermore, there is a need for the development of new aptamers beyond the therapeutic market. Assays and analytical systems, e.g. for medical diagnostics, but also for environmental analysis, offer a wide range of application. Some advantages of aptamers in comparison to antibodies, like the chemically synthesis of aptamers, their easy chemical

modification without loss of function, and their easier immobilization compared to antibody immobilization, should bring the replacement of antibody assays by aptamer based assays. Until now, only a limited number of aptamers are available for analytical application. A lot of publications describe the use of only a few individual aptamers in analytical systems as proof of principle (e.g. the thrombin aptamer in different biosensor configurations). Advances in the development of easy to use and automated SELEX procedures will encourage more researchers to develop additional aptamers for different targets. Provided there were more and various aptamers available, the chance for a better understanding of the optimum aptamer performance in assays and detection systems would be much higher. The gap between theory and practice has to be reduced as fast as possible (Ito and Fukusaki, 2004). Biologists have demonstrated that aptamers can do a lot of interesting things. Now it is up to technology development to produce more prototypes and get a deeper impact on industry (Mukhopadhyay, 2005).

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