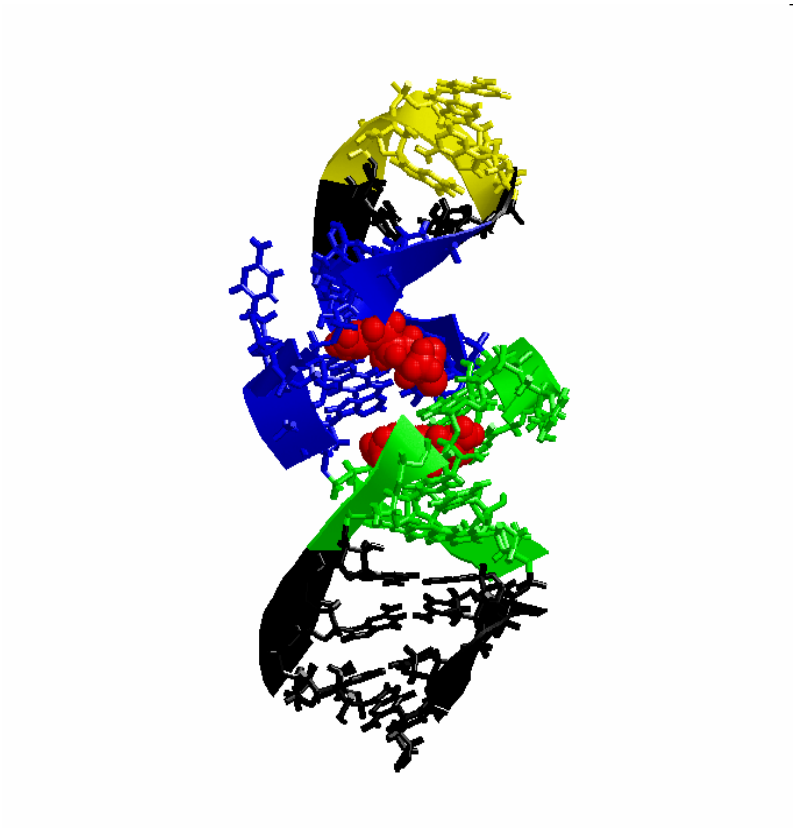
FOI
SWEDISH DEFENCE
RESEARCH AGENCY

FOI-R—1216--SE
April 2004
ISSN 1650-1942
Scientific report

Aptamers - Future tools for diagnostics and therapy

Karin Hjalmarsson, Anna Macellaro, Lena Norlander



NBC Defence
SE-901 82 Umeå

SWEDISH DEFENCE RESEARCH AGENCY
NBC Defence
SE-901 82 Umeå

FOI-R—1216--SE
April 2004
ISSN 1650-1942
Scientific report

Aptamers - Future tools for diagnostics and therapy

Karin Hjalmarsson, Anna Macellaro, Lena Norlander

Issuing organization	Report number, ISRN	Report type
FOI – Swedish Defence Research Agency NBC Defence SE-901 82 Umeå	FOI-R--1216--SE	Scientific report
	Research area code 3. NBC Defence and other hazardous substances	
	Month year April 2004	**Project no.** A438
	Customers code	
	Sub area code 32. Biological and Chemical Defence Research	

Author/s (editor/s)	Project manager
Karin Hjalmarsson Anna Macellaro Lena Norlander	Ann Göransson-Nyberg
	Approved by Åke Sellström
	Sponsoring agency Swedish Ministry of Defence
	Scientifically and technically responsible Karin Hjalmarsson

Report title

Aptamers - Future tools for diagnostics and therapy

Abstract (not more than 200 words)

The high target specificity and affinity make aptamers attractive tools for analytical, diagnostic or therapeutic purposes. Aptamers are also used as research tools to study protein functions and interactions as well as molecular mechanisms of diseases. Nucleic acid aptamers are selected from an oligonucleotide library (10^{15} sequences) by a selection scheme based on *in vitro* evolution principles (SELEX).

Since the introduction of the aptamer technology in 1990, the great interest in aptamers is reflected by the growing number of publications and patent applications. Aptamers have been selected to various targets and more than 1100 aptamer sequences, mostly RNA, are found in data bases. One of the few disadvantages of aptamers is the instability of the RNA molecule. Several approaches to solve the problem have been presented. For instance, by introducing modified bases the *in vivo* half-life was increased from minutes to hours. Other means of stabilization is the addition of sugars and by microencapsulation.

The majority of the patents and publications on aptamers concern diagnostic approaches. There are also many publications, which describe the isolation and medical effects of aptamers directed to critical targets in diseases. A few of these aptamers are evaluated in clinical trials.

Keywords

Aptamer, SELEX, diagnostics, therapy, analysis, review

Further bibliographic information	Language English
ISSN 1650-1942	**Pages** 42 p.
	Price acc. to pricelist

Utgivare	Rapportnummer, ISRN	Klassificering
Totalförsvarets Forskningsinstitut - FOI	FOI-R--1216--SE	Vetenskaplig rapport
NBC Skydd	**Forskningsområde**	
901 82 Umeå	3. Skydd mot NBC och andra farliga ämnen	
	Månad, år	**Projektnummer**
	April 2004	A438
	Verksamhetsgren	
	Delområde	
	32. B- och C- forskning	

Författare/redaktör	Projektledare
Karin Hjalmarsson	Ann Göransson-Nyberg
Anna Macellaro	**Godkänd av**
Lena Norlander	Åke Sellström
	Uppdragsgivare/kundbeteckning
	Försvarsdepartememtet
	Tekniskt och/eller vetenskapligt ansvarig
	Karin Hjalmarsson

Rapportens titel (i översättning)

Aptamerer - Framtida verktyg för diagnostik och terapi

Sammanfattning (högst 200 ord)

Aptamerers specificitet och affinitet till målmolekylen gör dem till attraktiva verktyg för analytiska, diagnostiska eller terapeutiska ändamål. De används även som forskningsverktyg för att studera proteinfunktioner och interaktioner såväl som sjukdomars molekylära mekanismer. Nukleinsyra aptemerer selekteras från ett oligonukleotidbibliotek (>10^{15} olika sekvenser) genom ett selektionsschema som bygger på de evolutionära principerna (SELEX).

Sedan aptamerteknologins introduktion 1990, har antalet publikationer och patentansökningar ständigt ökat. Aptamerer har selekterats fram mot varierande målmolekyler och mer än 1100 aptamersekvenser, mest RNA, finns i databaser. En av de få nackdelarna hos aptamerer är RNA molekylens instabilitet. Ett flertal förslag för att lösa problemet har presenterats. Genom att t ex introducera modifierade baser har aptamerens halveringstid utökats från minuter till timmar. Andra stabiliseringsmetoder som prövats är tillsats av sockermolekyler eller mikroinkapsling.

Huvuddelen av patent och publikationer rörande aptamer handlar om diagnostiska metoder. Det finns också många publikationer som beskriver isolering och medicinska effekter av aptamerer riktade mot kritiska sjukdomsfaktorer. Ett fåtal av dessa aptamerer utvärderas i klinisk prövning.

Nyckelord

Aptamer, SELEX, diagnostik, terapi, analys, review

Övriga bibliografiska uppgifter	**Språk** Engelska
ISSN 1650-1942	**Antal sidor:** 42 s.
Distribution enligt missiv	**Pris: Enligt prislista**

CONTENTS

1. Introduction

There is continued effort put into discovery of new substances for analysis, diagnosis and therapy to combat diseases and for environmental monitoring. An approach to search for novel pharmacological and diagnostic agents has emerged from the field of combinatorial chemistry. Pools or libraries of molecules are constructed and are subsequently screened for their pharmacological or diagnostic potential.

Aptamers[1] are a class of molecules, which has been of interest for about a decade. Aptamers are selected from combinatorial libraries and are typical single-stranded nucleic acids (DNA or RNA) between 30 and 70 nucleotides in length (molecular weight 9-20 kDa). There are also aptamers of a peptidic structure, peptide aptamers, but they will not be addressed further on. Hence, in this report aptamers refers to nucleic acid aptamers.

Since the introduction of the technology for isolation of nucleic acid aptamers in 1990, there has been a steady annual increase in the number of publications related to aptamers. During the last 10 years aptamers binding to for example cells, proteins, peptides, low molecular weight molecules (like antibiotics, amino acids, and dyes) or nucleic acids have been isolated. Aptamers display a high specificity and affinity for their target molecules, which make them suitable for a variety of applications. A continuously growing number of nucleic acid aptamers are used as research tools to study specific protein functions and interactions. Moreover, aptamers are used to study regulatory circuits and molecular mechanisms of disease processes. Aptamers can readily be adopted for analytical or diagnostic applications. *In vivo* experiments demonstrate that they generally exhibit low toxicity and immunogenicity characteristics, which make them suitable for therapeutical use. Technologies for the chemical synthesis of very large aptamer quantities have been developed, as have chemical modification protocols, to improve the stability and circulating half-life of the DNA and RNA molecules in blood plasma and other body fluids. The first aptamer-based therapeutics is now being tested in clinical trials.

The most efficient way to find aptamers is through a method utilizing nucleic acid libraries in conjunction with a selection scheme based on *in vitro* evolution principles. The process has been termed SELEX, standing for Systematic Evolution of Ligands by EXponential enrichment. Since DNA and RNA molecules adopt stable and intricately folded three-dimensional shapes they are capable of providing a scaffold for the interaction with functional side groups of a bound ligand. Randomised sequence pools of oligonucleotides can be synthesized with sequence complexities in the range of 10^{15} different molecules. This represents a structural diversity not matched by any other combinatorial technique and it is one of the prime advantages of the SELEX method.

In the study, we present a survey of the aptamer field and the development of aptamers for diagnostic, analytic and therapeutic purposes. The report reviews and evaluates the recent developments in the nucleic acid aptamer field.

[1] The word aptamer is derived from the Latin word *aptus* for “to fit”.

2. Aptamer Selection Techniques

2.1 The SELEX technique

In 1990 an *in vitro* selection technique was independently developed by two laboratories. By using the technique, specific binding nucleic acids could be isolated from pools of more than 10^{15} different molecules. The SELEX technique is today the overall method used for identification of specific binding aptamers to many different types of targets.

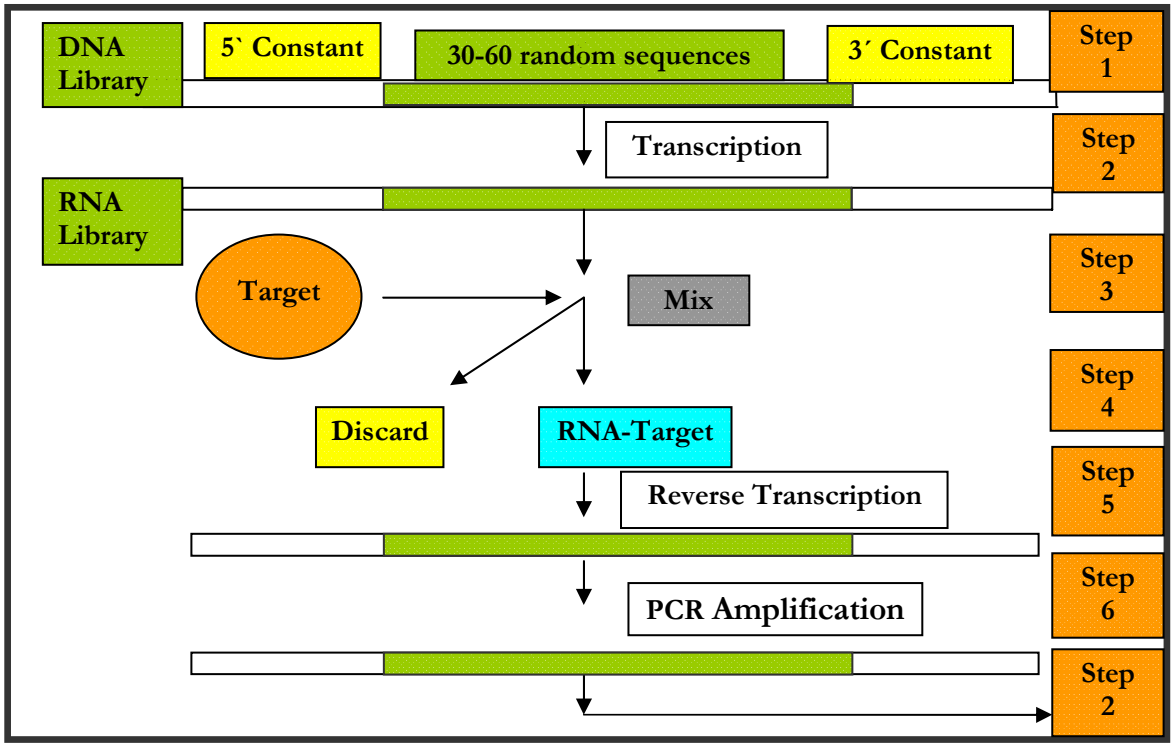


Figure 1. *In vitro* selection of nucleic acid aptamers by the SELEX technique. A synthetic library of single stranded DNA oligonucleotides contains a central region of 30-60 random nucleotides (potentially resulting in 10^{15}-10^{30} different molecules) with defined constant 5´and 3´regions, and defined primer binding sites (step 1). After conversion to double stranded DNA by reversed transcriptase PCR the DNA is transcribed *in vitro* using T7 RNA polymerase to provide a large library of RNA sequences (step 2). The RNA is allowed to fold into its specific three-dimensional structure and thereafter the target molecules are added to the pool. The rare aptamers, which have the specificity, bind the target (step 3). The unbound aptamers are discarded and the selected aptamers are recovered from the target (step 4). The eluted aptamers are reversed transcribed and PCR amplified (step 5-6). This new DNA-library is enriched for target-binding aptamers and constitutes the starting material for the next cycle (step 2). The cycle is repeated 5-25 times.

In short, the SELEX procedure (Figure 1) starts from a library of oligonucleotide sequences (>10^{15}), containing a random region flanked by defined constant 5´and 3´regions. The oligonucleotide library is converted to double stranded DNA, and transcribed *in vitro* to provide a large library of RNA sequences. Each of the RNA sequences in the pool will adopt a specific three-dimensional structure, as a result of sequence-determined intramolecular interactions. The target molecules are added to the aptamer pool in a buffer with conditions suitable for the target. The concentration of divalent ions in the buffer is important for the formation of nucleic acid secondary and tertiary structures. In the first cycle of selection there are few target-binding

aptamers and the method used for selecting binding from non-binding aptamers is crucial. The target-bound aptamers are eluted, reversed transcribed and PCR amplified, resulting in a new DNA-library. The next cycle is started from this library. This procedure is repeated in several cycles (typically 8-12), selecting for aptamers with increasing affinity and specificity. Negative selection steps are important to perform during the SELEX, because otherwise there is a risk of selection of non-specific aptamers. Successive selection and amplification cycles are performed until few sequence families dominate the population. These selected aptamers are finally cloned, sequenced and further characterised. DNA aptamers are selected in the same manner, with the exceptions that no reversed transcriptase step is needed and the strands have to be separated before the three dimensional structure is formed.

2.2 Selection methods

One of the most crucial aspects of the selection experiment is the partition of bound and unbound aptamer species, which requires the immobilisation or separation of the target or target-aptamer complex. There is a variety of selection methods, depending on the nature of the target and the possibilities available. Filtration using nitrocellulose filters was one of the first selection methods to trap a target-aptamer complex.[2] This method was successfully used to select ricin-binding aptamers.[3]

Centrifugation has also been used in the selection step. Aptamers binding to intact parasites could easily be collected by centrifugation.[4] A new elegant approach for selection was established, where the target molecule is bound to colloidal gold to confer a higher mass at the target, for further purification by centrifugation.[5]

A less common method is separation by molecular size on polyacrylamide gels (gel shift), where the complex is isolated from the gel and the aptamer is eluted and amplified.

However, separation on columns is an often used method, where the columns contain sepharose[6 7] or agarose[8]. The target can be modified to contain a tag, GST (glutathione-S-transferase) or His (6-8 residues linked to the N- or C-terminal of the protein), which binds to the column matrix.[9 10]

---

[2] Tuerk, C., and L. Gold. 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacterophage T4 DNA polymerase. Science 249:505-510.
[3] Hesselberth, J.R., *et al*. 2000. *In vitro* selection of RNA molecules that inhibit the activity of ricin A-chain. J. Biol. Chem. 275:4937-4942.
[4] Ulrich, H., *et al*. 2002. *In vitro* selection of RNA aptamers that bind to cell adhesion receptors of *Trypanosoma cruzi* and inhibit cell invasion. Braz. J. Med. Biol. Res. 34:295-300.
[5] Moreno, M., *et al*. 2003. Selection of aptamers against KMP-11 using colloidal gold during the SELEX process. Biochip. Biophys. Res. Commun. 308:214-218.
[6] Ellington, A.D., and J.W. Szostak. 1990. *In vitro* selection of RNA molecules that bind specific ligands. Nature 346:818-822.
[7] Leva, S., *et al*. 2002. GnRH binding RNA and DNA Spiegelmers: a novel approach toward GnRH antagonism. Chem. Biol. 9:351-359.
[8] Jhaveri, S.D., *et al*. 2000. Designed signalling aptamers that transducer molecular recognition to changes in fluorescence intensity. J. Am. Chem. Soc. 122:2469-2473.
[9] Martell, R.E., J.R., Nevins, and B.A. Sullenger. 2002 Optimizing aptamer activity for gene therapy applications using expression cassette SELEX. Mol. Ther. 6:130-134.
[10] Bae, S-J., *et al*. 2002. In vitro selection of specific RNA inhibitors of NFATc. Biochem. Biophys. Res. Commun. 298:486-492.

For selection of toxin-binding aptamers, conjugation of the target to magnetic beads has been used.[11] This selection method has also been useful in the development of an automated SELEX process.[12]

Table 1. Patented variations of the SELEX protocol.[13]

SELEX protocol	Application	Related US patents, No
Photo SELEX	Aptamers containing photoreactive groups capable of binding and/or photo cross-linking to and/or photo-activating a target molecule	5,763,177 6,001,577 6,291,184, 6,458,539
Chimeric SELEX	Combination of aptamers generated from two parent libraries	5,637,459
Blended SELEX	Combination of aptamers with other non-oligonucleotide groups such as peptides	5,683,867 6,083,696
Counter-SELEX	Method for generating aptamers that can discriminate between highly related molecules, such as theophylline and caffeine	5,580,737
Solution SELEX	Method for high efficiency partitioning between oligonucleotide with high and low affinity for a target molecule	5,567,588
Chemi-SELEX	Method for covalently linking an aptamer to its target	5,705,337
Tissue SELEX	Method for generating aptamers capable of binding to complex tissue targets such as collections of cells e.g. Tenascin-C in diseased tissue	5,789,157 6,376,474 6,232,071
Parallel SELEX (not strictly based on SELEX but adopts many of the basic principles)	Process using oligonucleotide ligands to facilitate binding with other reactant molecules, which can be screened against predetermined targets. The oligonucleotide bound to the selected reactant molecule can be amplified for further rounds of selection	5,858,660
Transcription-free SELEX	Describes a method for ligating random fragments of RNA bound to a DNA template to form the oligonucleotide library. As there is no transcription step more diverse nucleotides can be incorporated	6,387,620

Several variations of the SELEX protocol have been patented (Table 1). These protocols correspond to different useful methods for selection of aptamers and originate from the same research group at the University of Colorado, Boulder, USA. The earliest patent, from 1994, was in cooperation with the company NeXstar, which was later acquired by Gilead, Inc. The research group also cooperated with SomaLogic, Inc., on the development of Photo SELEX.

2.3 Stabilization of aptamers

Modification of single-stranded RNA may be performed for stabilization since the aptamers are sensitive to degrading enzymes. This is of special importance when the aptamer is used for therapy and the environmental parameters can not be modified. Degrading enzymes are common in body fluids as serum or urine. The stability is increased by changing the 2´-OH group of ribose to 2´-amino (NH_2) or 2´-fluoro (F)-groups on pyrimidines. Such modified nucleoside triphosphates function as substrate for T7 polymerase in *in vitro* transcription and may be included in the SELEX

[11] Bruno, J.G., and J.L Kiel. 2002. Use of magnetic beads in selection and detection of biotoxin aptamers by electrochemiluminescence and enzymatic methods. Biotechniques 32:178-180, 182-183.
[12] Cox, J.C., *et al*. 2002. Automated selection of aptamers against protein targets translated *in vivo*: from genes to aptamer. Nucleic Acids Res. 30:*e*108.
[13] Sampson, T. 2003. Protecting intellectual property rights in SELEX and aptamers. World Patent Information 25:343-349.

protocol.[14] An aptamer can also be protected from exonuclease degradation by capping its 3´-end after the SELEX process is completed.[15] Other ways of stabilization are by enlargement of the molecule through extended sequence[16] or by conjugating the aptamers to larger macromolecules like polyethylenglycol (PEG)[17].

Embedding into liposomes is another method to protect the aptamer. [18] Stabilized aptamers were coupled to a dialkylglyceol (DAG) moiety and inserted into the lipid bi-layer of liposomes. These aptamers have different orientations; some are directed inwards and others out into the surrounding solution.[19] Liposomes have also been used for delivery of aptamers, to improve the cellular uptake.[20]

Moreover, the Spiegelmer technology is useful to overcome the degradation problem. This technology is based on mirror image nucleic acid chemistry. The selection of Spiegelmers is performed by a modified SELEX method where an aptamer is selected against the mirror image of the natural target. After trimming, the equivalent "unnatural form" (L-form) of the aptamer is synthesised. Such a Spiegelmer binds both the natural target and the mirror target with high affinity and displays high resistance to enzymatic degradation compared with the natural D-form of the aptamer.[21 22]

2.4 Automated SELEX robotic systems

The expanding information on complete genome sequences and organismal proteomes raises the need for high throughput aptamer selection methods to identify aptamers to new targets, entire proteomes or metabolites.[23] The *in vitro* selection can be extremely time-consuming, from weeks to months. However, the introduction of robotic systems has made it possible to isolate aptamers by automated RNA selection.[24 25] These automated selection systems can be used in the discovery of new small molecule

[14] Micklefield, J. 2001. Backbone modification of nucleic acids: synthesis, structure and therapeutic applications. Curr. Med. Chem. 8:1157-1179.
[15] White, R.R., B.A. Sullenger, and C.P. Rusconi. 2000. Developing aptamers into therapeutics. J. Clin. Invest. 106:929-934.
[16] Hwang, B., and S-W. Lee. 2002. Improvement of RNA aptamer activity against myasthenic autoantibodies by extended sequence selection. Biochem. Biophys. Res. Commun. 290:656-662.
[17] Dougan, H., *et al*. 2000. Extending the lifetime of anticoagulant oligodeoxynucleotide aptamers in blood. Nucl. Med. Biol. 27:289-297.
[18] Brody, E.N., and L. Gold. 2000. Aptamers as therapeutic and diagnostic agents. J. Biotechnol. 74:5-13.
[19] Willis, M.C., *et al*. 1998. Liposome-anchored vascular endothelial growth factor aptamers. Bioconjug. Chem. 9:573-582.
[20] Mann, M.J., and V.J. Dzau. 2000. Therapeutic applications of transcription factor decoy oligonucleotides. J. Clin. Invest. 106:1071-1075.
[21] Leva, S., *et al*. 2002. GnRH binding RNA and DNA Spiegelmers: a novel approch toward GnRH antagonism. Chem. Biol. 9:351-359.
[22] Wlotzka, B., *et al*. 2002. In vivo properties of an anti-GnRH Spiegelmer: an example of an oligo-nucleotide-based therapeutic substance class. Proc. Natl. Acad. Sci. USA 99:8898-8902. Epub 2002, 17 June.
[23] Cox, J.C., *et al*. 2002. Automated acquisition of aptamer sequences. Comb. Chem. High Throughput Screening 4:289-299.
[24]Cox, J.C., and A.D. Ellington. 2001. Automated selection of anti-protein aptamers. Bioorg Med Chem 10:2525-2531.
[25] Cox, J.C., *et al*. 2002. Automated selection of aptamers against protein targets translated *in vivo*: from genes to aptamer. Nucleic Acids Res. 30: *e*108.

drugs as well as for selection of nucleic acid enzymes. [26] [27] The automated SELEX process has also been used for isolation of Spiegelmers in the purpose to identify drug candidates.[28]

[26] Burgstaller, P., A. Jenne, and M. Blind. 2002. Aptamers and aptazymes: accelerating small molecule drug discovery. Curr. Opin. Drug Discov. Dev. 5:690-700.
[27] Sooter, L.J., *et al*. 2001. Toward automated nucleic acid enzyme selection. Biol. Chem. 382:1327-1334.
[28] NOXXON Pharma AG, www.noxxon.net

3. Targets for selected aptamers

Aptamers can be selected for almost any type of substance. A wide variety of molecules have been targeted by *in vitro* selection experiments yielding specific aptamers.[29] Many small organic molecules with molecular weights of 100-1000 Da have been shown to be good targets for selection. A variety of aptamers are targeted against peptides or proteins, even those that do not normally interact with nucleic acid within their natural context. This includes antibodies, phospholipases, hormones and growth factors. Aptamers can also be selected against targets (such as toxins or prions), which are non-immunogenic or toxic. Furthermore, aptamers binding a variety of clinically important substances as well as micro-organisms and parasites have been isolated.

A compilation of more than 1100 sequences of aptamers is found in The Aptamer Database@The Ellington Lab.[30] The majority of aptamers, which have been described, are RNA molecules. Some examples of targets for isolated aptamers are shown in Table 2.

Table 2. Examples of targets for nucleic acid aptamers. Unless otherwise indicated, the examples have been taken from The Aptamer Database@The Ellington Lab.

		Nucleic acid type	
Target Type	**Target**	**DNA**	**RNA**
Inorganic	Zn^{2+}	-	X
	Malachite Green	-	X
	Vitamin B12	-	X
Nucleic	Adenine	-	X
	TAR element of HIV-1	X	X
	Yeast Phe-tRNA	-	X
Organic	MDA (4,4´-methylenedianiline)[31]	-	X
	L-Arginine	-	X
	Cocaine	X	-
	Sialyl Lewis X	-	X
	Tetracycline	-	X
Peptides	Rex-peptide of HTLV-1	-	X
	IFN-gamma	X	-
	Substance P	-	X
Proteins	Ricin A-chain	-	X
	RNase H, HIV-1 virus	X	-
	Tax protein, HTLV virus	-	X
	NF-kappa B	-	X
	Coagulation factor Factor VIIa	-	X
	G-protein coupled receptor of neurotensin, NTS-1	-	X
	Human acetylcholinesterase	-	X
	Myasthenic autoantibody mAB198	-	X
Others	Anthrax spores	X	-
	African trypanosomes	-	X
	Tumour microvessels (rat brain)	X	-

[29] Luzi, E., *et al*. 2003. New trends in affinity sensing: aptamers for ligand binding. Trends Anal. Chem. 22: 810-818.
[30] URL<http://aptamer.icmb.utexas.edu>.
[31] Brockstedt, U., *et al*. 2004. In vitro evolution of RNA aptamers recognizing carcinogenic aromatic amines. Biochem. Biophys. Res. Commun. 313:1004-1008.

4. Binding properties and structure of aptamers

Important characteristics for the success of analytical and diagnostic assays or therapy protocols based on aptamers are the affinity and the specificity of the nucleic acid that provides molecular recognition. Aptamers have shown a very high affinity and selectivity for their targets, with dissociation constants (K_ds) comparable to those of some monoclonal antibodies, sometimes even better. The K_d values of aptamers differ depending on the nature of the target substance. Normally, aptamers have K_ds between 10 nM and 10 pM for proteins, which are usually present in cells at concentrations between μM and nM. For small molecules, aptamers have K_ds between 10 μM and 10 nM (see Table 3). Small molecules are present in cells at concentrations far higher than protein concentrations, perhaps mM to μM.[32]

During the selection process there is an enrichment of aptamer sequences, based on binding of the target. This causes the three-dimensional structure of the aptamer complexes to reflect optimised scaffolds for specific target recognition. Aptamers bind their targets with high selectivity by discriminating closely related molecules from their targets on the basis of small structural changes. An example is a theofylline aptamer with a 10,000-fold lower affinity for caffeine, which differs in only a methyl group.[33]

The architecture of aptamer-target complexes is valuable for the study of molecular recognition processes. Furthermore, structural data on aptamer-target complexes will be especially helpful for the rational exploration and optimisation of new drug targets. Three-dimensional structures have been determined at high resolution for a number of aptamers in complex with their cognate ligands. Appendix 1 contains aptamer-ligand complexes for which structural data are available in the Protein Data Bank.[34]

Progress has been made in understanding the biophysical basis for the specificity of aptamer-target binding.[35] Nucleic acids are strongly negatively charged at physiological pH and many of the aptamer-target proteins are ligands for other acidic polymers. This has led to the assumption that aptamer-protein interactions are dominated by electrostatic interactions. The finding that electronegative pockets within the RNA fold were responsible for coordination of positively charged portions of the target molecule supports this. However, it has also been shown that 80 % of the binding energy was contributed by hydrogen bonding in an aptamer-ligand complex. Other forces, like stacking of aromatic rings and Van der Vaals shape complementarity, are also involved.

[32] Gold, L., *et al*. 2002. One, two, infinity: Genomes filled with aptamers. Chem. Biol. 9:1259-1264.
[33] Jenison, R.D., *et al*. 1994. High-Resolution Molecular Discrimination by RNA. Science 263:1425-142.
[34] Berman, H.M., *et al*. 2000. The Protein Data Bank. Nucleic Acids Res. 28: 235-242, URL<http://www.rscb.org/pdb>.
[35] James, W. 2001. Nucleic acid and polypeptide aptamers: a powerful approach to ligand discovery. Curr. Opin Pharmacol. 1:540-546.

Table 3. Examples of aptamers for which structural data are available in the Protein Data Bank.[36] The affinity constants (K_d) for aptamers are taken from Hermann and Patel, 2000, [37] unless otherwise indicated.

Target	Aptamer	Affinity, Kd (µM)	Pdb ID
ATP	DNA	~6 (AMP)	1AW4
FMN	RNA	~0,5	1FMN
Argininamide	DNA	~125	2ARG
Arginine	RNA	~60	1KOC
Malachite Green	RNA	~0,04 (TMR)[38A] ~0,8 (MG)[B]	1F1T
Citrulline	RNA	~65	1KOD
Vitamin B12	RNA	0,090[39]	1DDY
Theofylline	RNA	~0,3	1EHT
Biotin	RNA	~6[40]	1F27
Neomycin B	RNA	~0,115	1NEM
Tobramycin	RNA	~0,009	1TOB
Rex peptide, HTLV-1	RNA	~0,025	1C4J
Rev peptide, HIV-1	RNA	~0,004	1ULL
Tat protein, HIV	RNA	~0,0001	1NBK
Coat protein, MS2	RNA RNA	~0,002[41] ~0,082	5MSF 6MSF
Thrombin	DNA	~0,025	148D
Nucleolin	RNA	0,002-0,005[42]	1IE2

A; tetramethylrosamine and B; malachite green

One example of an aptamer-target complex that has been studied in detail is an RNA aptamer-Tat protein complex. [43] The K_d of this complex is very low, 0.1 nM. The sequence of the aptamer in comparison with the natural target suggested the existence of two binding sites. This was supported by the recently determined structure of the aptamer in complex with two argininamide molecules (see Figure 2). Two adjacent U:A:U base triples were formed (shown by arrows in Figure 2), widening the major groove to make space for the two argininamide molecules. The argininamide molecules bind to the G bases through hydrogen bonds and the binding is stabilized through stacking interactions.

[36] Berman, H.M., *et al*. 2000. The Protein Data Bank. Nucleic Acids Res. 28: 235-242, URL<http://www.rscb.org/pdb>.
[37] Hermann, T., and D.J. Patel. 2000. Adaptive recognition by nucleic acid aptamers. Science 287:820-825.
[38] Baugh, C., D. Grate, and C. Wilson. 2000. 2.8 Å crystal structure of the malachite green aptamer. J. Mol. Biol. 301:117-128.
[39] Sussman, D., J.C. Nix, and C. Wilson. 2000. The structural basis for molecular recognition by the vitamin B12 RNA aptamer. Nat. Struct. Biol. 7:53-57.
[40] Nix, J., D. Sussman, and C. Wilson. 2000. The 1.3 A crystal structure of a biotin-binding pseudoknot and the basis for RNA molecular recognition. J. Mol. Biol. 296:1235-1244.
[41] Parrott, A.M., *et al*. 2000. RNA aptamers for the MS2 bacteriophage coat protein and the wild-type RNA operator have similar solution behaviour. Nucleic Acids Res. 28:489-497.
[42] Bouvet, P., *et al*. 2001. Recognition of pre-formed and flexible elements of an RNA stem-loop by nucleolin. J. Mol. Biol. 309:763-775.
[43] Matsugami, A., *et al*. 2003. Structural basis of the highly efficient trapping of the HIV Tat protein by an RNA aptamer. Structure 11:533-545.

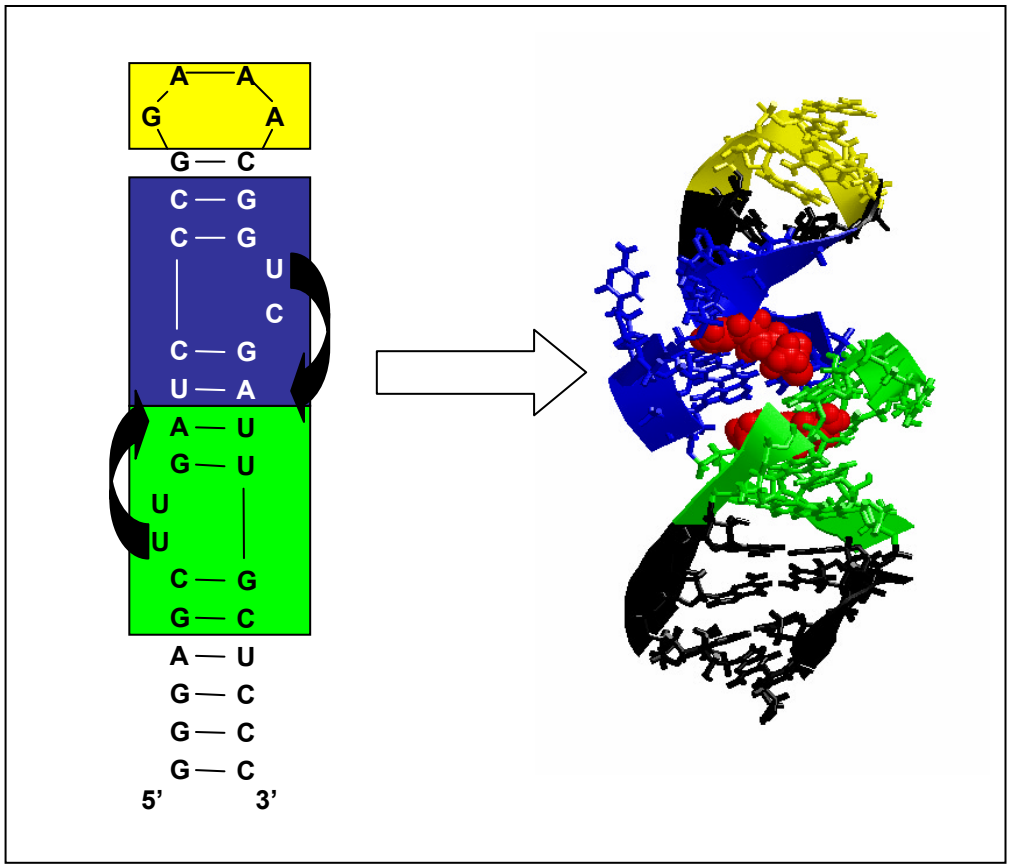


Figure 2. Sequence and folding of the RNA aptamer binding the Tat protein of HIV-1 and the three-dimensional structure of the aptamer in complex with two argininamide molecules (in red). The aptamer has two binding sites (in green and blue). The loop at the top of the hairpin is marked in yellow. The sequence and folding of the RNA aptamer is from Matsugami, A. *et al*[43] and the three-dimensional structure of the aptamer in complex with argininamide was generated using the computer programme Ras Top 2.0 (Philippe Valadon, 2000-2002) and structural data from Protein Data Bank (PDB Id: 1NBK).

5. Diagnostic and Therapeutical Use

A growing number of aptamers are being described as scientific and biotechnological tools for studying specific cellular protein functions and protein-ligand interactions. They are also used to decipher biologically relevant regulatory circuits or to improve understanding of molecular mechanisms of several disease processes. An increasing number of proteins involved in cell growth have been found to be altered through multiple mechanisms of oncogene activation and there is an obvious interest in aptamers directed to these proteins. Aptamers have also been selected to targets which have a suspected or verified role in diseases in the brain, eyes and kidneys as well as in arthritis, inflammatory, cardiovascular and autoimmune diseases. Molecules able to bind tightly and specifically to the surface of sick cells would greatly benefit diagnosis and treatment of many diseases. Whereas antibodies have the ability to specifically recognize for example tumour cell markers, large size and immunogenicity often limit their pharmacological value. Aptamers are new promising alternatives to antibodies in diagnostics.

Improved knowledge of infectious agents and the interaction between antibiotics and their RNA targets are other examples of studies in which aptamers are of great value. The following chapter gives several examples on aptamers used as research tools, as diagnostic or analytical tools and in various medical applications.

5.1 Aptamers as Research Tools

Pathway elucidation

Aptamers can be used as specific inhibitors to dissect intracellular signalling and transport pathways. An example of this kind is an RNA aptamer, which acts at sub-nanomolar levels as an inhibitor of a specific mitogen-activated protein (MAP) kinase.[44] In mammalian cells, different MAPKs are involved in specifying responses which include cell proliferation, apoptosis, and differentiation. Inappropriate activation of specific MAPK pathways is associated with various disease states in humans (including cancer and autoimmune diseases).

Another example is an RNA aptamer acting as a nanomolar inhibitor of the transcription factor NF-κB that works by blocking the interaction with the DNA recognition site.[45] Inhibition of NF-κB-dependent gene activation could be a valuable strategy for enhancing the activities of antiviral and anticancer agents.

G-protein-coupled receptors (GPCRs) are integral membrane proteins involved in signal transduction and constitute major drug targets for disease therapy. Aptamers could represent a valuable tool to probe the role of such receptors in normal tissue and disease pathology and for co-crystallization with receptors for structure determination by X-ray crystallography. As an example of this strategy, aptamers directed against the rat neurotensin receptor NTS-1 were isolated.[46] One of the aptamers was

[44] Seiwert, S., *et al*. 2000. RNA aptamers as pathway-specific MAP kinase inhibitors. Chem. Biol. 7:833-843.
[45] Lebruska, L.L., and L.J. Maher. 1999. Selection and characterization of an RNA decoy for transcription factor NF-κB. Biochemistry 38:3168-3174.
[46] Daniels, D.A., *et al*. 2002. Generation of RNA aptamers to the G-protein-coupled receptor for neurotensin, NTS-1. Anal. Biochem. 305: 214-226.

characterized in detail and shown to bind to both the rat receptor and the human receptor with nanomolar affinity. The aptamer was also demonstrated to interact with rat neurotensin receptors expressed in membrane preparations and in intact cells. Another example is the isolation of a nuclease-resistant RNA aptamer directed against Neuropeptide Y in order to characterize the interaction between the peptide and its receptors.[47] Neuropeptide Y is widely distributed in the central and peripheral nervous system and modulates a range of physiological processes.

One of the many targets for which aptamers have been selected is the protein tyrosine phosphatase group, the PTPases. The PTPase YOP51 of *Yersinia pestis* has been found to be a virulence determinant.[48] An aptamer to the PTPase gene Yop51 was shown to specifically inhibit the dephosphorylation activity. This anti-PTPase aptamer is useful for investigating the role of the enzyme and also as a possible therapeutical tool.

Tools in studies of diseases

Prions are thought to be involved in the pathogenesis of transmissible spongiform encephalopathies (TSEs) such as scrapie, bovine spongiform encephalopathy (BSE), and Creutzfeldt-Jakob disease. According to the protein-only hypothesis, the crucial step in prion propagation is the conversion of PrP^{C} into its structural and infectious isoform PrP^{SC}. The biochemical processes involved in prion propagation, prion processing, and prion protein aggregation are largely unknown. Aptamers for infectious prions have been isolated in order to investigate conformational aspects of prion pathogenesis. [49] [50] One of these aptamers has more than 10-fold higher affinity for PrP^{SC} than for recombinant PrP^{C} and inhibits the accumulation of abnormal PrP in a cell-free conversion assay. This aptamer could provide a lead for the development of diagnostic and therapeutic approaches to TSEs.

Aptamers can also be used to examine the pathogensis of infectious agents. A high affinity single stranded DNA aptamer population against the kinetoplastid membrane protein-11 (KMP-11) of *Leishmania infantum* has been selected by a novel SELEX methodology using colloidal gold. This protein is a major component of the cell membrane of the parasites and it has been suggested to be involved in mobility or in some other aspects of the flagellar structure. The aptamer population has been characterized in a series of *in vitro* experiments, suggesting that it may be used as a powerful tool to further investigate the role of KMP-11 during *Leishmania* development and as a diagnostic tool for the infection.[51]

Aptamers and antibiotics

One way of studying the mode of action of RNA-binding antibiotics is to determine the structure of RNA-antibiotic complexes. The natural target sites of RNA-binding antibiotics are often too large for structural characterization. Two methods of reducing

[47] Proske, D., *et al*. 2002. A Y2 receptor mimetic aptamer directed against Neuropeptide Y. J. Biol. Chem. 277:11416-11422.
[48] Bell, S.D., *et al.* 1998. RNA Molecules That Bind to and Inhibit the Active Site of a Tyrosine Phosphatase. J. Biol. Chem. 273:14309-14314.
[49] Weiss, S., *et al*. 1997. RNA aptamers specifically interact with the prion protein PrP. J. Virol. 71:8790-8797.
[50] Rhie, A., *et al*. 2003. Characterization of 2'-fluoro-RNA aptamers that bind preferentially to disease-associated conformations of prion protein and inhibit conversion. J. Biol. Chem. 278:39697-39705.
[51] Moreno, M., *et al*. 2003. Selection of aptamers against KMP-11 using colloidal gold during the SELEX process. Biochem. Biophys. Res. Commun. 308:214-218.

the RNA length are dissection of the natural target RNA to that of the antibiotic RNA-binding domain or isolation (via *in vitro* selection) of small RNAs that bind specifically to antibiotics. Aptamers are perfect tools for studying the interaction of small ligands with RNA. RNA aptamers that bind to antibiotics include binders to tetracycline,[52] tobramycin,[53] lividomycin,[54] kanamycin B,[55] moenomycin A,[56] neomycin B,[57] streptomycin,[58] spectinomycin,[59] the peptide antibiotic viomycin,[60] and chloramphenicol[61].

Tools for purification

Affinity chromatography is a powerful method for rapid selection and purification of compounds from complex mixtures. The affinity and specificity of aptamers is now sufficient for them to be used as affinity chromatography media.

Two DNA aptamers were covalently linked to fused silica capillary columns to serve as stationary-phase reagents in capillary electrochromatography. Separations of binary mixtures of amino acids (D-trp and D-tyr), enantiomers (D-trp and L-trp), and polycyclic aromatic hydrocarbons (naphthalene and benzo[a]pyrene) were achieved.[62] Moreover, aptamers immobilized in fused silica columns have been used to affinity purify the protein L-selectin.[63]

Aptamers have also been used for separation of adenosine and analogues.[64] A biotinylated-DNA aptamer that binds adenosine and related compounds in solution was immobilized by reaction with Streptavidin. The Streptavidin was covalently attached to porous chromatographic supports. The aptamer medium was packed into fused-silica capillaries to form affinity chromatography columns. It was demonstrated that the column could selectively retain and separate cyclic-AMP, NAD^+, AMP, ADP, ATP, and adenosine, even in complex mixtures such as tissue extracts.

---

[52] Berens, C., A. Thain, and R. Schroeder. 2001. A tetracycline-binding RNA aptamer. Bioorg. Med. Chem. 9:2549-2556.
[53] Jiang, L., and D.J Patel. 1998. Solution structure of the tobramycin-RNA aptamer complex. Nat. Struct. Biol. 5:769-774.
[54] Lato, S.M., and A.D Ellington. 1996. Screening chemical libraries for nucleic-acid-binding drugs by in vitro selection: a test case with lividomycin. Mol. Divers. 2:103-110.
[55] Kwon, M., *et al*. 2001. In vitro selection of RNA against kanamycin B. Mol. Cells 11:303-311.
[56] Schurer, H., *et al*. 2001. Aptamers that bind to the antibiotic moenomycin A. Bioorg. Med. Chem. 9:2557-2563.
[57] Cowan, J.A., *et al*. 2000. Recognition of a cognate RNA aptamer by neomycin B: quantitative evaluation of hydrogen bonding and electrostatic interactions. Nucleic Acids Res. 28:2935-2942.
[58] Tereshko, V., E. Skripkin, and J. Patel Dinshaw. 2003. Encapsulating streptomycin within a small 40-mer RNA. Chem. Biol. 10:175-187.
[59] Zimmerman, J.M., and L.J. Maher. 2002. In vivo selection of spectinomycin-binding RNAs. Nucleic Acids Res. 30:5425-5435.
[60] Wallis, M.G., *et al*. 1997. In vitro selection of a viomycin-binding RNA pseudoknot. Chem. Biol. 4:357-366.
[61] Burke, D.H., *et al*. 1997. RNA aptamers to the peptidyl transferase inhibitor chloramphenicol. Chem. Biol. 4:833-843.
[62] Kotia, R.B, L. Li, and L.B. McGown. 2000. Separation of nontarget compounds by DNA aptamers. Anal. Chem. 72:827-831.
[63] Romig, T., C. Bell, and D. Drolet. 1999. Aptamer affinity chromatography: combinatorial chemistry applied to protein purification. J. Chromatogr. 731:275-284.
[64] Deng, Q., *et al*. 2001. Retention and separation of adenosine and analogues by affinity chromatography with an aptamer stationary phase. Anal. Chem. 73:5415-5421.

5.2 Diagnosis and Analysis

Diagnosis of infectious diseases

Aptamers are new alternatives to antibodies in the diagnostics of pathogenic organisms. Aptamers directed against spores of *Bacillus anthracis* vaccine strains Sterne and A 16R have been isolated and characterized.[65] [66] Furthermore, a patent has been published concerning an aptamer chip, containing spore-binding aptamers. At binding of spores to the aptamer chip an electric and/or photochemical change occurs, giving a specific signature.[67]

A lot of attention has been focused on developing new technology for diagnosis and treatment of infections caused by the Hepatitis C virus. A number of aptamers directed to various targets of this virus have been reported. One of the targets is the non-structural protein 3, which has two distinct activities, as a protease and a helicase. Binding of the aptamer inhibited the protease activity. [68] The level of inhibition in cells was increased four-fold by construction of a tandem chimeric aptamer.

Hepatitis C virus RNA replicase has been identified in patient's blood using an RNA aptamer immobilized on magnetic bead and analyzed by MALDI-TOF mass spectrometry.[69]

Aptamers directed to toxins

Recently, DNA aptamers directed to the Cholera toxin and the Staphylococcal Enterotoxin B (SEB) have been isolated.[70] The main purpose was to use these aptamers as tools for detection. SEB is involved in several severe disease patterns and it has been used as a target for the generation of biologically stable mirror-image aptamers, Spiegelmers.[71] Since the full-length protein is not accessible to chemical peptide synthesis, a stable domain of 25 amino acids was identified as a suitable selection target. The isolated Spiegelmer bound the whole protein target with only slightly reduced affinity and had a dissociation constant of 420 nM. These data also demonstrate the possibility to identify Spiegelmers against large protein targets by a domain approach.

Verotoxins are produced by Enterohemorrhagic *Escherichia coli*, and are classified into two types based on their different amino acid sequences. Verotoxin-1 is the same toxin as the Shiga toxin produced by *Shigella dysenteriae* type 1, and is called the

---

[65] Bruno, J.G., and J.L. Kiel. 1999. In vitro selection of DNA aptamers to anthrax spores with electro-chemiluminiscence detection. Biosens. Bioelectron. 14:457-464.
[66] Zhen, P., *et al*. 2001. Structure and affinity analysis of aptamers to Bacillus anthracis spores. Jiefangjun Yixue Zazhi 26:419-422.
[67] US Patent 6303316, October 2001, filed in June 2000. *Organic semiconductor recognition complex and system*. Inventors: J.L. Kiel *et al*.
[68] Nishikawa, F., *et al*. 2003. Inhibition of HCV NS3 protease by RNA aptamers in cells. Nucleic Acids Res. 31:1935-1943.
[69] Cho, S., *et al*. 2001. A micro bead affinity chromatography for hepatitis C virus (HCV) RNA replicase detection using RNA aptamer in blood. Micro Total Analysis Systems 2001, Proceedings μTAS 2001 Symposium, 5th, Monterey, CA, US, Oct. 21-25, 2001. Eds: Ramsey, J. M., A. van den Berg. Publisher: Kluwer Acad Publ, Netherlands. pp 601-602.
[70] Bruno, J.G., and J.L. Kiel. 2002. Use of magnetic beads in selection and detection of biotoxin aptamers by electrochemiluminescence and enzymatic methods. Biotechniques 32:178-180.
[71] Purschke, W.G., *et al*. 2003. DNA Spiegelmer to staphylococcal enterotoxin B. Nucleic Acids Res. 31:3027-3032.

Shiga-like toxin. Recently, a DNA aptamer directed against the toxin was isolated.[72] A high affinity ligand capable of selectively recognizing Verotoxin-1 can be advantageously used for detection and quantitative determination of the toxin.

Microcystin is a toxin generated from the Cyanobacteria causing water blooms. A DNA aptamer showing specific binding to this toxin has been isolated. The direct detection range using an immobilized aptamer and a surface plasmon resonance biosensor was 50-1000 μg/ml.[73]

The first example of an anti-toxin aptamer was an RNA aptamer directed to ricin. The ricin toxin inhibits protein synthesis by interfering with the translation process. The toxin is a ribosome-inactivating protein and disables translation by depurinating a conserved site in eucaryotic rRNA. An aptamer, which was generated to the catalytic ricin A-chain, inhibited the toxin.[74] The binding efficiency was improved by truncating the original 80 nucleotides aptamer to a 31 bases oligonucleotide. There is, however, no publication so far (January 2004) on any application of the anti-ricin aptamer. Another ribosome-inactivating protein, Pepocin, has also been the target for RNA aptamer selection.[75]

Diagnosis of brain diseases

Alzheimer's disease is correlated with the deposition of amyloid peptides in the brain of the patients. The amyloid is thus a major target in the search for novel diagnostic and therapeutic approaches. High-affinity RNA aptamers against the amyloid peptide have been isolated from a combinatorial library and the binding of the RNA to the amyloid fibrils was confirmed by electron microscopy.[76] The chemical synthesis of these nucleic acids enables tailor-made modifications. By introduction of specific reporter groups these RNAs can become suitable tools for analytical and diagnostic purposes.

Diagnosis of cardiovascular diseases

Elevated total homocysteine in plasma or serum is used as a diagnostic marker of certain forms of cardiovascular disease. S-adenosylhomocysteine (SAH) is a key intermediate in the metabolism of the amino acid methionine and also the direct and only source of homocysteine. An RNA aptamer binding SAH with an affinity and selectivity comparable to that of a monoclonal antibody and with a high diagnostic potential has been isolated.[77]

Aptamers for imaging

A particular application for which aptamers might be well suited is diagnostic imaging. A DNA aptamer binding to the enzyme neutrophil esterase, present in

[72] United States Patent Application 20030119159, filed in July 2002. Aptamer capable of specifically adsorbing to Verotoxin-1 and method for obtaining the aptamer. Inventors: Okada, K., *et al*.
[73] Nakamura, C., *et al*. 2001. Usage of a DNA aptamer as a ligand targeting microcystin. Mol. Cryst. Liq. Cryst. 371:369-374.
[74] Hesselberth, J.R., *et al*. 2000. In vitro selection of RNA molecules that inhibit the activity of Ricin A Chain. J. Biol. Chem. 275:4937-4942.
[75] Hirao, I., *et al*. 2000. RNA aptamers that bind to and inhibit Ribosome-inactivating protein, Pepocin. J. Biol. Chem. 275:4943-4948.
[76] Ylera, F., *et al*. 2002. Selection of RNA aptamers to the Alzheimer's disease amyloid peptide. Biochem. Biophys. Res. Commun. 290:1583-1588.
[77] Gebhart, K., *et al*. 2000. RNA aptamers to S-adenosylhomocysteine: Kinetic properties, divalent cation dependency, and comparison with anti-S-adenosylhomocysteine antibody. Biochemistry 39:7255-7265.

neutrophil granules at sites of inflammation, has been isolated. It was tested for this application in an *in vivo* rat model with promising result.[78] The aptamer was labelled with ^{99m}Tc, a γ-emitting radionuclide with a half-life of 6 hours. Animals receiving the aptamer were imaged on a γ-camera at intervals during 4 hours.

Neoangiogenesis, the formation of new blood vessels, is a key feature of tumour development and it is associated with quantitative and qualitative changes in expression of endothelial proteins. Such molecules could serve as molecular addresses differentiating the tumour vasculature from those of the normal brain. Transformed endothelial cells have been used as a complex target to select DNA aptamers.[79] One of 25 aptamers analyzed bound selectively microvessels of brain glioblastoma but not the vasculature of the normal brain. The molecular target protein of the aptamer was isolated from endothelial cells.

Aptamers in analysis

Aptamers have been introduced into analytical chemistry in a variety of applications, such as various modes of capillary electrophoresis, affinity chromatography, and biosensing, as exemplified below. Other protein-DNA/RNA binding assays have also been used to study binding interactions and the stoichiometry of affinity complexes, and, to a lesser extent, in the determination of target analytes in biological systems.[80]

In addition, aptamers have been applied in affinity probe capillary electrophoresis for rapid determination of low concentrations of IgE, thrombin and HIV-1 reverse transcriptase.[81] [82]

Although it is clear that aptamers could be synthesized and put on solid surfaces, it is less clear how aptamer-target binding could be detected most conveniently. Several different approaches have been taken to address this problem.[83]

Detection principle

One possibility to detect aptamer-ligand binding exploits the ability to link aptamers directly or indirectly to protein enzymes and thereby use them as specific stains. This approach has been used to detect bile salts with sensitivity in the micromolar range.[84] A 5'-biotin-labeled DNA oligomer with a 40-nucleotide length that is defined by the *in vitro* selection method was connected with alkaline phosphatase through an avidin-biotin linkage and applied to an enzyme immunoassay format.

Alternatively, the aptamer can be fused to an RNA enzyme (ribozyme) to produce an "aptazyme". The binding of the ligand to the aptazyme allosterically activates the

[78] Charlton, J., J. Sennello, and D. Smith. 1997. *In vivo* imaging of inflammation using an aptamer inhibitor of human neutrophil esterase. Chem. Biol. 4:809-816.
[79] Blank, M., *et al*. 2001. Systematic evolution of a DNA aptamer binding to rat brain tumour microvessels. selective targeting of endothelial regulatory protein pigpen. J. Biol. Chem. 276:16464-16468.
[80] Pavski, V., and X.C. Le. 2003. Ultrasensitive protein-DNA binding assays. Curr. Opin. Biotechnol 14:65-73.
[81] German, I., D.D, Buchanan, and R.T. Kenndy. 1998. Aptamers as ligands in affinity probe capillary electrophoresis. Anal. Chem. 70:4540-4545.
[82] Pavski, V., and X.C. Le. 2001. Detection of Human Immunodeficiency Virus type 1 reverse transcriptase using aptamers as probes in affinity capillary electrophoresis. Anal. Chem. 73:6070-6076.
[83] James, W. 2001. Nucleic acid and polypeptide aptamers: a powerful approach to ligand discovery. Curr. Opin. Pharmacol. 1:540-546.
[84] Kato,T., *et al*. 2000. Bioassay of bile acids using an enzyme-linked DNA aptamer. Analyst 125:1371-1373.

enzymatic function which is detected by a suitable substrate.[85] Allosteric ribozymes have been made that responds to small organic compounds, oligonucleotides, proteins, and metal ions.[86]

An alternative way to detect binding is by using fluorescence. If an aptamer is modified by introducing a fluorescent moiety into the ligand-binding site, significant changes in fluorescence on binding can be readily detected.[87] Slightly more promising is the use of fluorescence dequenching, in which a fluorophore linked to one end of an aptamer is quenched by a second group linked to the other end but adjacent in the three-dimensional structure. When the ligand binds, the conformational rearrangement of the aptamer removes the quencher from the fluorochrome, resulting in an easily detected signal. This made it possible to achieve real time detection of low amounts of target .[88] [89] [90]

Recently, a new strategy for fluorescence detection was reported. It involves designing aptamer-based fluorescent reporters that function by switching structures from DNA-DNA duplex to DNA-target complex. The duplex is formed between a fluorophore-labeled DNA aptamer and a small oligonucleotide modified with a quenching moiety (denoted QDNA). When the target is absent, the aptamer binds to QDNA, bringing the fluorophore and the quencher into close proximity for maximum fluorescence quenching. When the target is introduced, the aptamer prefers to form the aptamer-target complex. The switch of the binding partners for the aptamer occurs in conjunction with the generation of a strong fluorescence signal owing to the dissociation of QDNA.[91]

The oncoprotein platelet-derived growth factor (PDGF) and its isoforms play important roles in the cell transformation process and in tumour growth and progression. In view of this, ultrasensitive assays have been developed for PDGF, two of which involve the use of aptamers. In one of the assays a fluorescein-labelled single-stranded DNA aptamer was used.[92] In the other assay, so-called proximity ligation was used.[93] In the assay, PCR is applied for detection of target-bound aptamers. The method has a 1000-fold linear range and can detect zeptomole (10^{-21}) levels, corresponding to 24,000 molecules or approximately 1 fg of protein, of PDGF (Appendix 3). The detection limit is comparable with the Origen

[85] Hesselberth, J., *et al*. 2000. *In vitro* selection of nucleic acids for diagnostic applications. J. Biotechnol. 74:15-25.
[86] Breaker, R.R. 2002. Engineered allosteric ribozymes as biosensor components. Curr. Opin. Biotechnol. 13:31-39.
[87] Jhaveri, S.D., *et al*. 2000. Designed signalling aptamers that transduce molecular recognition to changes in fluorescence intensity. J. Am. Chem. Soc. 122:2469-2473.
[88] You, K.M., *et al*. 2003. Aptamers as functional nucleic acids: In vitro selection and biotechnological applications. Biotechnol. Bioprocess Eng. 8:64-75.
[89] Yamamoto, R., T. Baba, and P.K. Kumar. 2000 Molecular beacon aptamer fluorescense in the presence of Tat protein of HIV-1. Genes Cells 5:389-396. (Erratum in: Genes Cells 2000 5:423.)
[90] Hamaguchi, N., A. Ellington, and M. Stanton. 2001. Aptamer beacons for the direct detection of proteins. Anal. Biochem. 294:126-131.
[91] Nutiu, R., and Y. Li. 2003. Structure-switching signaling aptamers. J. Am. Chem. Soc. 125:4771-4778.
[92] Fang, X., *et al*. 2001. Molecular aptamer for real-time oncoprotein platelet-derived growth facor monitoring by fluorescens anisotropy. Anal. Chem. 73:5752-5757.
[93] Fredriksson, S., *et al*. 2002. Protein detection using proximity-dependent DNA ligation assays. Nature Biotechnol. 20:473-477.

electrochemoluminiscence immunoassay, which has a detection limit for the toxin SEB of 1 pg/ml corresponding to 0.04 pM.[94]

Biosensors based on aptamers

Aptamers have been used extensively in biosensing applications. Aptamers are well suited to application in biosensors to specifically detect a large variety of molecules like, for example, proteins, metabolites, amino acids, and nucleotides. Aptamers can also be utilized when the targets, such as toxic agents or non-immunogenic substances, are hard to address by using antibody sensors.

The first biosensor using an aptamer was designed for measurements of L-adenosine.[95] An RNA aptamer directed against L-adenosine was attached through an avidin-biotin bridge to the core of a multimode fiber. The sensor indicates binding of FITC-labeled L-adenosine and real-time fluorescence measurements are performed by the competition with the L-adenosine (unlabeled) of the sample in the micromolar range.

A quartz crystal biosensor designed to detect concentrations and ligand affinity parameters of free unlabeled proteins in real time has been developed.[96] Using a model system with human IgE as the analyte and single-stranded DNA aptamers or an anti-IgE antibody as immobilized ligands, it could be demonstrated that aptamers are equivalent to antibodies in terms of specificity and sensitivity. Both receptor types selectively detected 0.5 nM of IgE. In addition, the aptamer receptors tolerated repeated affine layer regeneration after ligand binding and recycling of the biosensor with little loss of sensitivity. Because of the small size and nonprotein nature of the aptamers, they were immobilized in a dense, well-oriented manner, thus extending the linear detection range to 10-fold higher concentrations of IgE. In addition to demonstrating that an aptamer-based biosensor can specifically and quantitatively detect an analyte in various complex protein mixes, the aptamer-ligand proved to be relatively heat resistant and stable over several weeks.

Several biosensors have been designed for measurements of thrombin. The first aptamer-based biosensor that could be used to detect free and nonlabelled non-nucleic acid targets was a thrombin-biosensor.[97] A more recent example is a fiber-optic biosensor using an aptamer receptor.[98] An antithrombin DNA aptamer was immobilized on the surface of silica microspheres, and these aptamer beads were distributed in microwells on the distal tip of an imaging fiber. A different oligonucleotide bead type, which was prepared using the same method, the aptamer beads was also included in the microwells to measure the degree of nonspecific binding. The imaging fiber was coupled to a modified epifluorescence microscope system, and the distal end of the fiber was incubated with a fluorescein-labelled thrombin (F-thrombin) solution. Nonlabelled thrombin could be detected using a competitive binding assay with F-thrombin. The aptamer beads selectively bound to

[94] Kijek, T.M., *et al*. 2000. Rapid and sensitive immunomagnetic-electrochemiluminiscent detection of staphylococcal enterotoxin B. J. Immunol. Methods 236: 9-17.
[95] Kleinjung, F., *et al*. 1998. High-affinity RNA as a recognition element in a biosensor. Anal. Chem. 70:328-331.
[96] Liss, M., *et al*. 2002. An aptamer-based quartz crystal protein biosensor. Anal. Chem. 74: 4488-4495.
[97] Potyrailo, R.A., *et al*. 1998. Adapting selected nucleic acid ligands (aptamers) to biosensors. Anal. Chem. 70:3419-3425.
[98] Lee, M., and D.R Walt. 2000. A fiber-optic micro array biosensor using aptamers as receptors. Anal. Biochem. 282:142-146.

the target and could be reused without any sensitivity change. The fiber-optic microarray system has a detection limit of 1 nM for nonlabelled thrombin, and each test can be performed in about 15 minutes including the regeneration time.

A fluorescence aptamer-biosensor for cocaine has been developed.[99] An instability was engineered in one stem of a three-way junction that forms the cocaine-binding pocket and the resulting short stem was end labelled with a fluorophore and a quencher. In the absence of cocaine, two stems are open, but in its presence they close and the three-way junction forms. This major structural change brings fluorophore and quencher together thereby signalling the presence and concentration of ligand. The sensor is selective for cocaine over its metabolites, can operate in serum, and could be useful for the screening of cocaine hydrolases.

Protein arrays based on aptamers

High-throughput protein arrays allow the miniaturized and parallel analysis of large numbers of diagnostic markers in complex samples. Protein microarrays are constructed from recombinantly expressed, purified, and yet functional proteins, entailing a range of optimized expression systems. Antibody microarrays are becoming a robust format for expression profiling of whole genomes. Alternative systems, such as aptamer arrays, are at proof-of-concept stage.[100] One of the attractions of nucleic acid aptamers is that they could be applied to high-throughput analytical techniques because they can be synthesized chemically, immobilized on a range of supports and are sufficiently robust to be repeatedly denatured and refolded. Microarrays based on aptamers are robust and have long shelf-life. In a recent publication, a biosensor array based on fluorescently labelled aptamers was described.[101] It demonstrated specific detection and quantitation of cancer-associated proteins (inosine monophosphate dehydrogenase II, vascular endothelial growth factor, basic fibroblast growth factor) in the context of human serum and in cellular extracts.

Arrays can also be constructed based on light-sensitive photoaptamers, containing 5-halo-uracil. These aptamers capture proteins in the sample solution, and the chips are then exposed to ultraviolet light, covalently cross-linking the aptamers with their target proteins. The chips are washed vigorously to remove all unbound protein, and the captured proteins are revealed using a universal protein stain.

High sensitivity and specificity of two modified DNA photoaptamers capable of photocross-linking recombinant human basic fibroblast growth factor (bFGF) has been demonstrated.[102] The aptamers exhibited high sensitivity for bFGF comparable with commercially available ELISA monoclonal antibodies with an absolute sensitivity of at least 58 pg bFGF under prevailing test conditions. The aptamers exquisitely distinguished bFGF from other proteins.

Photoaptamers have been selected for the proteins thrombin and bFGF. The sensitivity, specificity, and crosslinking efficiency of photoaptamers directed to

[99] Stojanovic, M.N., P. de Prada, and D.W Landry. 2001. Aptamer-based folding fluorescent sensor for cocaine. J. Am. Chem. Soc. 123:4928-4931.
[100] Walter, G., *et al*. 2002. High-throughput protein arrays: prospects for molecular diagnostics. Trends Mol. Med. 8:250-253.
[101] McCauley, T., N. Hamaguchi, and M. Stanton. 2003. Aptamer-based biosensor array for detection and quantification of biological macromolecules. Anal. Biochem. 319:244-250.
[102] Golden, M.C., *et al*. 2000. Diagnostic potential of PhotoSELEX-evolved ssDNA aptamers. J. Biotechnol. 81:167-178.

thrombin and bFGF indicated their suitability as capture agents for multiplexed microarrays.[103]

A key question regarding the use of photoaptamer probes is the specificity of the cross-linking reaction. The specificities of three photoaptamers have been explored by comparing their reactions with target proteins and non-target proteins.[104] The range of target/non-target specificity varies from 100- to $>10^6$-fold with most values $>10^4$-fold. The contributions of the initial binding step and the photocross-linking step were evaluated for each reaction. Photocross-linking never degraded specificity and significantly increased aptamer specificity in some cases. Moreover, the application of photoaptamer technology to proteomics was investigated in microarray format. Immobilized anti-HIV-gp120 aptamer was able to detect sub-nanomolar concentrations of target protein in 5 % human serum. The levels of sensitivity and specificity displayed by photoaptamers, combined with other advantageous properties of aptamers, should facilitate development of protein chip technology.

5.3 Therapy

Aptamers specific for various signalling targets and cancer therapy

VEGF

The vascular endothelial growth factor (VEGF) is an example of a factor involved in tumour growth. It has been shown to stimulate the growth of vessels into the tumours, which are growing more rapidly. Another example is the VEGF stimulation of growth of new vessels in the eye which cause age-related sight defects. RNA aptamers, which specifically bind and inactivate VEGF *in vitro*, have been isolated. The effect of an anti-VEGF aptamer was initially tested in monkeys with introduced eye defects. The test showed that the aptamer retained activity after 28 days in the vitreous body, but that the half-time was 94 hours. No toxicological effect was noted in the eye and no antibody formation occurred. Based on the results the authors recommended a dose of 1-2 mg aptamer per eye per month.[105] A clinical study (presented in 2002) on humans with injection of anti-VEGF-aptamers in the eye showed that 80 % of the patients (15) retained or improved the eye sight and they had no side-effects.[106] The VEGF aptamer has also been successfully used for the inhibition of diabetic retinal leukostasis and blood-retinal barrier breakdown in both early (72,4 % and 82,6 %) and established (48,5 % and 55,0 %) diabetes.[107] Results from a large Phase III clinical trial (i.e. the final test before marketing the drug) using an experimental drug were recently presented.[108] About 1200 patients have been injected with an anti-VEGF drug into the eyes. The results indicate that the aptamer, which prevents blood vessel growth, slows vision loss but does not improve vision in most patients.

---

[103] Zichi, D., *et al*. 2002. Photoaptamer technology: development of multiplexed microarray protein assays. Clin. Chem. 48:1865-1868.
[104] Smith, D., *et al*. 2003. Sensitivity and specificity of photoaptamer probes. Mol. Cell. Proteomics 2:11-18.
[105] Drolet, D.W., *et al*. 2000. Pharamacokinetics and safety of an anti-vascular endothelial growth factor aptamer (NX1838) following injection into the vitreous humor of Rhesus monkeys. Pharm. Res. 17:1503-1510.
[106] Anonymus. 2002. Preclinical and phase 1A clinical evaluation of an anti-VEGF pegulated aptamer (EYE001) for the treatment of excudative age-related macular degeneration. Retina 22:143-152.
[107] Susumu, I., *et al*. 2003. VEGF164 is proinflammatory in the diabetic retina. Invest. Ophthalmol. Vis. Sci. 44:2155-2162.
[108] www.news.harvard.edu/gazette/2003/11.20/14-eye.html (2004-01-02).

In order to develop a controlled drug delivery system a microsphere containing the anti-VEGF RNA aptamer was constructed.[109] The aptamer was stabilized by the addition of the disaccharide trehalose before lyophilization and encapsulation. The aptamer-containing microspheres were packed into a device and placed on the orbital surface of the sclera. The aptamer was continually released with an average rate of 2 μg/ day over a period of 20 days and retained its activity.

PDGF

Aptamers have been selected to the platelet-derived growth factor (PDGF). This growth factor is involved in many diseases and is thus an attractive target for therapeutic aptamers. Rats with the renal disease progressive mesangioproliferative glomerulonephritis were treated within a week after disease introduction with an anti-PDGF aptamer.[110] A significant reduction of mesangioproliferative changes was observed.

The PDGF has a role also in the development of chronic pulmonary hypertension. Experiments in an animal model show that treatment with an aptamer directed to PDGF reduced the development of muscular thickening of small pulmonary arteries by 47-66 %.[111]

Anticoagulant therapy

The goals of anticoagulant therapy in cardiovascular diseases are to inhibit fibrin deposition and platelet aggregation. There is a search for new antithrombotics to be used in addition to the established drugs, i.e. aspirin, heparin. Several of the proteins in the coagulase cascade have been targets for DNA and RNA aptamer selection performed during the last decade. Some recent published examples are given. Thrombin is a coagulation protease involved in a multitude of physiological process. DNA aptamers have been prepared to the two main binding sites of thrombin, the fibrin-binding site exosite-1 and the heparin-binding site exosit-2. The studies have so far been limited to *in vitro* interaction experiments and biodistribution and stability in mice models in order to assess the clinical potential.[112] [113] An alternative target for the development of an anticoagulant is the coagulation factor IXa and an aptamer directed to this factor has also been isolated.[114]

Inflammatory disorders

Inflammatory diseases and artherosclerosis have been associated with pro-inflammatory mediators. Such mediators may be potentially therapeutic targets and have been target for aptamer selection. The monocyte chemo-attractant protein 1 (MCP-1) is a powerful pro-inflammatory mediator. A series of RNA aptamers that

[109] Carrasquillo, K., *et al*. 2003. Controlled delivery of the anti-VEGF aptamer EYE001 with poly (lactic-co-glycolic) acid microspheres. Invest. Ophtalmol. Vis. Sci. 44:290-299.
[110] Ostendorf, T., *et al*. 2001. Specific antagonism of PDGF prevents renal scarring in experimental glomerulonephritis. J. Am. Soc. Nephrol. 12:909-918.
[111] Balasubramaniam, V., *et al*. 2003. Role of platelet-derived growth factor in vascular remodelling during pulmonary hypertension in the ovine fetus. Am. J. Physiol. 284:L826-833.
[112] Dougan, H., *et al*. 2000. Extending the lifetime of anticoagulant oligodeoxynucleotide aptamers in blood. Nucl. Med. Biol. 27:289-297.
[113] Boncler, M.A., M. Koziolkiewicz, and C. Watala. 2001. Aptamer Inhibits Degradation of Platelet Proteolytically Activated Receptor, PAR-1, by Thrombin. Thromb. Res. 104:215-222.
[114] Rusconi, C., *et al*, 2002. RNA aptamers as reversible antagonists of coagulation factor IXa. Nature 419:90-94.

bind specifically to MCP-1 have been isolated.[115] These aptamers have high specificity for MCP-1 and is capable of antagonizing the function of the protein by binding to various targets on the protein.

Oncostatin M is another proinflammatory mediator involved in inflammatory disorders. RNA aptamers, which have been isolated to oncostatin, reduce efficiently (in the nanomolar range) the binding to its receptor.[116] One of these aptamers has been truncated in length to 33 bases and, in order to stabilize the aptamer, all pyrimidin bases and 14 out of 18 purines have been substituted with 2´-fluorine and 2´-O-methyl, respectively. This truncated nuclease-resistant aptamer is an attractive therapeutic candidate for treatment of rheumatoid arthritis.

Auto-antibodies in autoimmune diseases

Conventional treatment for the autoimmune diseases is limited to relatively non-specific suppression of the immune system. It is desirable to target the pathogenic autoantibodies without interfering with other parts of the immune system. Aptamers have been selected in order to be used in the treatment of such diseases. Myasthenia gravis is a neuromuscular disease which is caused by the formation of autoantibodies directed to the acetylcholin receptors located in the postsynaptic muscle cell membrane. Aptamers with specificity to autoantibodies isolated from patients have been developed. [117] These aptamers protect the receptors on human cells from the autoantibodies and are hot candidates for future treatment of the disease.

In systemic lupus erythematosus (SLE) patients, high levels of autoantibodies directed to DNA mediate many of the clinical manifestations of lupus. Recently, an RNA aptamer with high specificity to a monoclonal anti-DNA autoantibody has been isolated.[118] This aptamer strongly binds to the autoantibody that was used as selection target, but not to other similar autoantibodies, i.e. it binds to the epitope binding site of the antibody. As a consequence, DNA binding to the autoantibody is efficiently inhibited by the aptamer. The effective inhibitory concentrations for RNA aptamer was in a nanomolar range rather than a micromolar range shown for peptides previously isolated against this type of antibodies. The RNA aptamer will be further developed for a therapeutic approach.

Brain diseases

The critical event in the pathophysiology of transmissible spongioform encephalities appears to be the conversion of the cellular protein PrP into the abnormal isoform PrP^{SC}. The human form of the disease is called Creutzfeltd-Jacob´s Syndrome and it is extremely rare (1 case in 10^6 persons). The disease is characterized by the formation of protein aggregates which accumulate in the central nervous system. Two separate groups have recently presented aptamers with specificity for a domain which is functionally important for the conversion of PrP to PrP^{SC}. Both aptamers were shown to reduce the proportion of PrP^{SC} in neuroblastoma cells and in a physiological cell-

[115] Rhodes, A., *et al*. 2001. The generation and characterisation of antagonist RNA aptamers to MCP-1. FEBS Lett. 506:85-90.
[116] Rhodes, A., *et al*. 2000. The generation and characterization of antagonist RNA aptamers to human oncostatin M. J. Biol. Chem. 275:28555-28561.
[117] Hwang, B., and S-W Lee. 2002. Improvement of RNA Aptamer Activity against Myasthenic Autoantibodies by Extended Sequence Selection. Biochem. Biophys. Res. Commun. 290:656-662.
[118] Young-Mee, K., *et al*. 2003. Specific modulation of the anti-DNA autoantibody-nucleic acids interaction by the high affinity RNA aptamer. Biochem. Biophys. Res. Commun. 300:516-523.

free conversion system, respectively.[119] [120] These aptamers could be potentially drugs for prion-protein associated diseases, but this would require a mean for transport across the blood brain barrier.

Infectious diseases

Aptamers are attractive candidates as drugs for treatment of infectious diseases caused by viruses or parasites. Parasitic diseases are among the most devastating illnesses in the world. They cause the suffering of hundreds of millions of people as well as an unknown number of wild and domestic animals. The problem is amplified by the fact that the number of drugs for treating parasite infections is very small.

Chaga´s disease is caused by the parasite *Trypanosoma cruzi*, which multiply intra-cellularly. Aptamers which are directed to the organism´s binding site on the cell, were selected. By competing for the receptor these aptamers inhibited the binding and invasion of Trypanosomas in monkey kidney cells.[121] An alternative approach is presented in two separate publications where aptamers are intended to be transporters for harmful molecules. One research group has focused on aptamers directed to conserved structural epitopes of the variant surface glycoproteins of Trypanosomas.[122] These epitopes are not accessible to antibodies but can be accessed by smaller molecules. The selected aptamers are able to recognize different glycoprotein variants and bind to the surface of the organisms. The idea is to couple antibodies to these aptamers, which will direct the antibodies to the various Trypanosomas variants. The alternative approach is aptamers selected to a surface protein exclusively expressed by the parasites in the infective blood stream.[123] These aptamers are able to penetrate into a flagellar pocket of the parasite where they recognize the target protein. After binding the target, the aptamer is endocytosed and transported to the lysosome. It was shown that also a biotin-aptamer complex was transported to the lysosome. It is presumed that this mechanism could be used to "piggy-back" RNA-coupled toxins to the lysosome and thus to inhibit the growth of the organism. Aptamers directed against the parasite *Trypanosoma brucei* have also been isolated.[124]

An important issue in infectious medicine is to find drugs for treatment of virus infections. One of the most urgent research areas concern anti-HIV drugs, i.e. treatment for AIDS. A series of aptamers, which interfere with virus multiplication in human cells, has been developed. The aptamers interact with various components involved in virus propagation, i.e. HIV reverse transcriptase (RT)[125], integrase[126], Env

[119] Proske, D., *et al*. 2002. Prion-protein-specific aptamer reduces PrPSc formation. Chembiochem 3:717-725.
[120] Rhie, A., *et al*. 2003. Characterisation of 2´-fluoro-RNA aptamers that bind preferentially to disease-associated conformations of prion protein and inhibition conversion. J. Biol. Chem. 278:39697-39705.
[121] Ulrich, H., *et al*. 2002. *In vitro* Selection of RNA Aptamers that bind to Cell Adhesion Receptors of *Trypanosoma cruzi* and Inhibit Cell Invasion. J. Biol. Chem. 277:20756-20762.
[122] Lorger, M., *et al*. 2003. Targeting the variable surface of African trypanosomes with variant surface glycoprotein-specific, serum-stable RNA aptamers. Eucaryotic. Cell. 2:84-94.
[123] Homann, M., and H.U. Goringer. 2000. Uptake and intracellular transport of RNA Aptamers in African trypanosomes suggest therapeutic "Piggy-Back" approach. Bioorg Med Chem 9:2571-2580.
[124] Göringer, H.U., M. Homann, and M. Lorger. 2003. In vitro selection of high-affinity nucleic acid ligands to parasite target molecules. Int. J. Parasitol. 33:1309-1317.
[125] Pheroze, J., and V.R. Prasad. 2002. Potent inhibition of human immunodeficiency virus type 1 replication by template analog reverse transcriptase inhibitors derived by SELEX. J. Virol. 76:6545-6547.
[126] De Soltrait, V.R., *et al*. 2002. DNA aptamers derived from HIV-1 RNase H inhibitors are strong anti-integrase agents. J. Mol. Biol. 324:195-203.

structural protein[127], nucleocapsid protein[128], and prevent the formation of intact virus particles. The RNA aptamer is encapsidated into the virions and they have a strong reduced infectivity. The integrase is a retroviral-encoded enzyme, which is involved in the integration of the viral genome in the host DNA. The aptamers to the HIV structural proteins Env and the capsid protein inhibit assembly of virus particles.

The Cytomegalovirus (CMV) is another group to which aptamers have been selected.[129] CMV is a prevalent viral pathogen mainly experienced in inapparent infections. Anti-CMV aptamers bind viruses with high affinity *in vitro* and effectively inhibited infection in cell culture. The two aptamers are directed to the virus glycoprotein B and H, respectively, and by binding they block virus entry.

Aptamers for targeting of agents

Aptamers have been selected to bind tumour-associated membrane antigens.[130] Two distinct aptamers bound to a prostate-specific membrane antigen, PSMA. The aptamers did not share any consensus sequence and bound to distinct epitopes of PSMA. Such aptamers are attractive candidates as carriers of imaging agents and therapeutic agents directed to prostate cancer cells.

Tenascin-C (TN-C) is an extracellular matrix protein that is overexpressed during tissue remodelling processes, including tumour growth. Anti-TN-C aptamers that are related in sequence have been isolated using both TN-C and TN-C-expressing glioblastoma cells.[131] A size-minimized and nuclease-stabilized aptamer, TTA1, binds to the fibrinogen-like domain of TN-C. This 13 kDa aptamer is intermediate in size between peptides and single chain antibody fragments, both of which are superior to antibodies for tumour targeting because of their smaller size. TTA1 defines a new class of ligands that are intended for targeted delivery of radioisotopes or chemical agents to diseased tissues.

Treatment of drug addicts

Abused drugs, such as cocaine, inhibit the nicotinic acetylcholine receptor. There is a need for substances which can compete with the cocaine binding site without affecting the channel-opening equilibrium. Two classes of RNA aptamers, which bind the receptor, have been found.[132] Both classes of aptamers compete with cocaine for the target site on the receptor but only one of them does not affect the normal function of the receptor.

---

[127] Bai, J., *et al*. 2002. RNA-Based Anti-HIV-1 Gene Therapeutic Constructs in SCID-hu Mouse Model. Mol. Ther. 6:770-782.
[128] Kim, S.J., *et al*. 2002. Selection and Stabilization of the RNA Aptamers against the Human Immunodeficiency Virus Type-1 Nucleocapsid Protein. Biochem. Biophys. Res. Commun. 291:925-931.
[129] Wang, J., H. Jiang, and F. Liu. 2000. In vitro selection of novel RNA ligands that bind human cytomegalovirus and block viral infection. RNA 6:571-583.
[130] Lupold, S.E., *et al*. 2002. Identification and characterization of nuclease-stabilized RNA molecules that bind human prostate cancer cells via the prostate-specific membrane antigen. Cancer Res. 62:4029-4033.
[131] Hicke, B.J., *et al*. 2001. Tenascin-C aptamers are generated using tumour cells and purified protein. J. Biol. Chem. 276:48644-48654.
[132] Ulrich, H., *et al*. 1998. In vitro selection of RNA molecules that displace cocaine from the membrane-bound nicotinic acetylcholine receptor. Proc. Natl. Acad. Sci. USA 95:14051-14056.

6. Commercial Interest in Aptamer Technology

The use of aptamers in a variety of areas from diagnostics to therapy is reflected in the growing number of applications of aptamer technology. Some companies, such as Archemix in the USA, have been founded on the technology while others, as for example Glaxo Wellcome plc och Eli Lilly & Co, have licensed the technology. Some of the companies active in the aptamer technology area are listed in Table 4.

Table 4. Examples of companies active in the aptamer technology area.

Company and Country	Area
Aptamera, Inc.[133] USA	Aptamera is a life science product company with new therapeutic and diagnostic technologies targeting cancer.
Archemix, Inc.[134] USA	Archemix develops aptamers and riboreportersTM (allosteric ribozymes that couple recognition to detection) for therapeutics and drug discovery. Using novel nucleic acid platforms, Archemix builds mechanism-based drug discovery programmes around virtually any target. During 2001-2004 Archemix has entered into collaboration with or licensed technology to Nuvelo, Inc (ARC183), SomaLogic, Inc. (broad use of aptamers), Aptamera, Inc. (AGRO100), Regado Biosciences (discovery and development), Noxxon Pharma AG (technology), and Ribozyme Pharmaceuticals, Inc. (proteomics, molecular diagnostics).
Genta Inc.[135] USA	Genta Inc. is a biopharmaceutical company focused on the identification, development and commercialization of drugs for the treatment of patients with cancer. In their DNA/RNA medicine area they employ antisense and decoy aptamer technology.
Gilead Sciences, Inc.[136] USA	Gilead research and development programmes focus on nucleotides, nucleosides, liposomes, durable HIV inhibitors and other small molecules. Gilead has several compounds in various stages of clinical development. In 1999, Gilead acquired the company NeXstar Pharmaceuticals, Inc. In 2001 Gilead out-licensed property rights under the SELEX process patent to Archemix, Inc.
Isis Innovation Ltd[137] UK	Isis Innovation is a wholly-owned subsidiary of the University of Oxford, founded to exploit know-how arising out of research. Isis Innovation holds patents involving aptamers binding to prion protein (PrP), CD4, gp120, and streptavidin. In 2001 the US company VITEX signed a license agreement with Oxford University granting exclusive rights to Oxford´s proprietary RNA ligand technology, designed to selectively bind to prions.
NOXXON Pharma AG[138] Germany	NOXXON´s objective is the development of a new generation biopharmaceuticals, using its unique Spiegelmer (chemically synthesized mirror-inverted nucleic acids, either RNA or DNA) technology. NOXXON holds patents for aptamers binding to, for example, gonadotropin releasing hormone and the toxin SEB.
SomaLogic, Inc.[139] USA	SomaLogic is developing novel proteomics systems and applications based on its proprietary photoaptamer technology. This technology provides the basis for a new approach to multiplexed protein measurement. In 2003 SomaLogic entered agreements with Beckman Coulter, Inc (development of technology), National Cancer Institute (development of aptamers aginst proteins), Merial Ltd (diagnostics for BSE), and Mitsui & Co (distribution in Asia).

Several of the applications of aptamers exemplified in this report are covered by patents. The majority of researchers and companies active in the aptamer technology area are from the USA. In January 2004 there were listed 107 aptamer-related patents

[133] www.aptamera.com
[134] www.archemix.com
[135] www.genta.com
[136] www.gilead.com
[137] www.isis-innovation.com
[138] www.noxxon.net
[139] www.somalogic.com

(1993-2004) and 51 patent applications (2001-2003) in the USPTO data bases.[140] Examples of patents from the USPTO and EPO[141] databases from year 2003 to present time are shown in Appendix 2. As can be seen in the appendix the majority of the patents are from the USA. The patents are issued for applications concerning development of SELEX technology, aptamers to specific proteins, binding assays utilising aptamers, diagnostics, and drug-delivery systems utilising aptamers.

In January 2004 there were two aptamers in different phases of clinical trials. The aptamer EYE001 (NX1838, Macugen), an angionesis inhibitor, is currently in Phase III clinical trials. The aptamer was developed by Gilead Inc. and has been out-licensed to EyeTech Pharmaceuticals. Aptamera Inc.'s aptamer AGRO100, is in Phase I clinical trial for treatment of advanced solid tumours. AGRO100 binds to nucleolin protein on cancer cell surfaces, causing arrest of the cell cycle, inhibits DNA replication, and initiates apoptosis.

Archemix Inc.'s first drug development candidate ARC183, an anti-thrombin aptamer, is expected to enter clinical trials in 2004 and Genta Inc.'s aptamer decoy binding the protein CRE, a transcription factor, has passed preclinical evaluation and is expected to enter clinical trials. The latter aptamer binds to and blocks the protein complexes that would normally turn on genes regulated by CRE, thereby inhibiting cancer cell growth.

So far, there are no diagnostic or analytical product utilising aptamers out on the market. However, SomLogic Inc. has products in late development and has entered collaboration with Beckman Coulter to integrate the photoaptamer technology with an automatic work station.

[140] US Patent & Trademark Office, URL<http://www.uspto.gov/>. Search for patents or applications with "aptamer", "SELEX"or "nucleid acid ligand" in abstract.
[141] European Patent Office, URL<http://ep.espacenet.com/>.

7. Summary and Concluding Remarks

Since the introduction in 1990 of the SELEX technology for isolating aptamers there has been an increasing interest in the area. As shown in Figure 3, the annual number of publications has enhanced by a factor of 2 only the last 3 years. In addition, there is a steady increase in the number of patents filed for aptamer sequences or use of aptamers in different applications. Why this interest for nucleic acid aptamers?

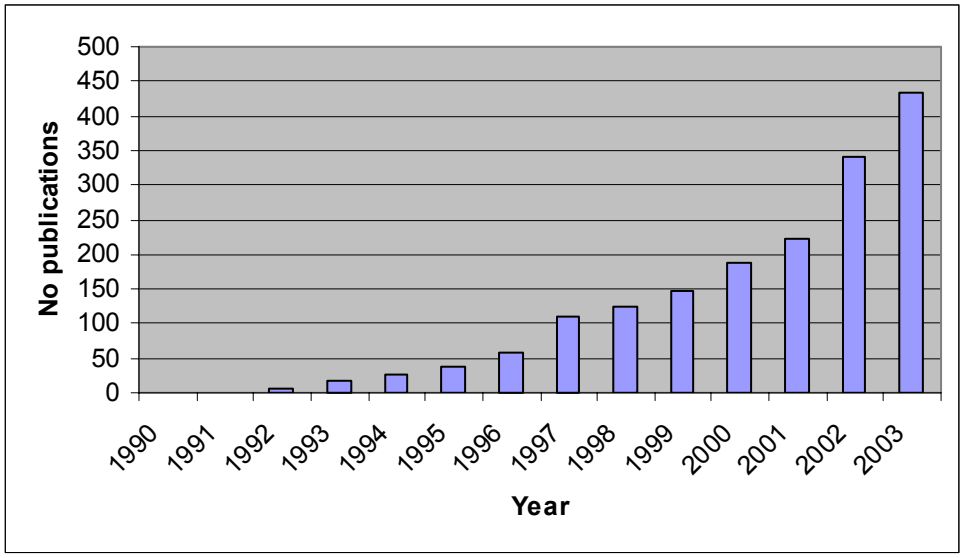


Figure 3. Annual number of aptamer-related publications 1990-2003. Based upon a database search in Biosis, Medline and Chemical abstracts.

Current techniques or products for analysis, diagnosis, therapy and environmental monitoring have limitations. Due to this, there is a continuous search for new substances for a variety of applications.

Aptamers show great promise based on several characteristic features. They can be raised in a rapid and efficient way against a broad range of target substances (low to high molecular weights, inorganic to organic substances). The aptamers display a high specificity and affinity to their targets, comparable to monoclonal antibodies and sometimes even higher. They are isolated through an *in vitro* procedure (SELEX), which increases the flexibility in selection conditions and opens up the possibility for adjustment depending on the final application of the aptamer. Automated procedures are under development, which will further increase the rapidity by which an aptamer or aptamers against one or more targets could be isolated. After the first aptamer has been isolated it can easily be produced in large-scale by chemical synthesis, which also makes it possible to introduce modifications for stabilization or introduction of reporter molecules. The variation between the successive batches is low and comparable to the production of monoclonal antibodies. The large-scale synthesis, furthermore, reduces the cost of the final product. Finally, aptamers normally display no immunogenicity or toxicity as could be the case for other substances.

In applying aptamers into different applications several problems have been encountered and addressed. Nucleic acids have a natural sensitivity to nucleases and this hampers the development of aptamers for various medical applications. For this reason, a lot of effort has addressed the development of methods for introducing modified bases into the aptamer during or after *in vitro* synthesis. It is also possible to

couple the aptamer to large molecules like PEG to increase the stability. By efforts like this the half-life of aptamers, in for example human serum, has been increased from minutes to days. The desired durability in the body will of course vary depending on the clinical picture.

One of the draw-backs of aptamers is a limited chemical diversity since only four different nucleotides build up the aptamer, compared to 20 amino acids for peptides. However, by introducing modified nucleotides in the synthesis the diversity may be increased.

Metal ions, especially divalent ions, normally have a profound effect on nucleic acid conformation and signalling. This could be of a larger importance in applications of aptamers for therapy compared to analysis or diagnosis, where there is more control over assay conditions. However, if the final use of the aptamer is known from the start it is possible to adjust the selection conditions to suit the coming application.

Aptamers, as nucleic acids, are negatively charged at physiological pH and many of the proteins targeted by aptamers are ligands for acidic polymers. This indicates that aptamers preferentially bind to positively charged portions of a target molecule and thus, there is a limitation of raising aptamers to some targets or regions of targets. This is of less importance in analytical or diagnostic applications where the primary aim is to develop a specifically binding molecule. On the contrary, in a therapeutical application usually not only binding is desired but also the inhibition of a target molecule (e.g. the aptamer has to bind to a specific, biological active region of the target molecule). This is a limitation also encountered by other selection procedures and substances. For instance, antibodies are usually directed to hydrophilic, surface-exposed portions of proteins.

As demonstrated in Chapter 5 and Appendix 3 of this report, aptamers have been widely used in the development of analytical and diagnostic applications. The majority of the publications and patents, which were found in this survey of the aptamer field, deal with diagnostic approaches. Aptamers have been introduced into protein-DNA/RNA binding assays, various modes of capillary electrophoresis, affinity chromatography, and biosensing. There are several reasons for this. Firstly, aptamers can be selected for almost any type of substance (see Table 2, Chapter 2). Small organic molecules have been shown to be good targets for selection as well as peptides or proteins, even those that do not normally interact with nucleic acid within their natural context. As an example, the first isolated aptamers were directed against adenosine (Mw 267 Da) and thrombin (Mw 34,000 Da).

Secondly, aptamers are new alternatives to antibodies in assays of different kinds. Aptamers can be raised against targets for which there are dificulties in obtaining an antibody, i.e. due to small size, non-immunogenicity or toxicity of the target molecule. The aptamers have high specificity and affinity, comparable to monoclonal antibodies and sometimes even better. Another advantage is that production of aptamers, in contrast to antibodies, does not involve animals. Moreover, in comparison with antibodies or other proteinaceous compounds, the aptamers have a high stability and this enables the use in analytical applications which greatly reduces background noise.

Thirdly, the small size of aptamers enables higher density stationary phases in for instance affinity chromatography. Moreover, they allow novel approaches for immobilization based on oligonucleotide chemistry.

One early problem that was addressed concern the detection of binding between aptamer and target. Several different approaches have been taken to solve this problem. Most of the published methods utilise different variations of fluorescence.

In the post-genomic time there is an interest for techniques enabling simultaneous measurement of a number of substances in complex samples. Microarray technologies fulfil this need for analysis of expression profiling on both transcriptional and protein level. Up to now, antibody-based microarrays are becoming a robust format for protein expression profiling of whole genomes. Aptamer-based arrays could constitute an alternative system. One of the attractions of aptamers is that they could be applied to high-throughput analytical techniques, because they can be synthesized chemically, immobilized on a range of supports and is sufficiently robust to be repeatedly denatured and refolded.

One interesting development in the protein chip technology area is arrays based on light-sensitive photoaptamers. These aptamers capture proteins in a sample solution, the aptamer-target complex is covalently cross-linked, and washed vigorously before revealing the captured protein on the chip. The photocross-linking has been shown not to influence the specificity. On the contrary, in some cases a significant increased specificity was observed.

The sensitivities or detection limits (shown in Appendix 3) of aptamer-based analytical methods ranges from 0.04 pM (PDGF) to 10 μM (cocaine) with the majority in the lower nanomolar range. For the most sensitive method the detection signal was amplified by the use of aptamers in conjunction with PCR. The sample volumes are in the range 0.1-20 μl and the time needed for an analysis varies from seconds to minutes. The detection limits are as good as, or even better than, other types of analytical methods. Usually, enzyme-based sensors have detection limits in the range 0.1 μM to 0.1 mM. Optical DNA biosensors can reach as low as 0.2 pM. Chemical analysis for low molecular weight compounds by ion chromatography or capillary electrophoresis has detection limits in the range of nM to μM.

Aptamers have properties, which make them promising tools for therapy and a large variety of targets with crucial role in diseases have been addressed for this purpose. The majority of the examples are, however, reports of the successful isolation of target-specific aptamers and the demonstration of their efficiency in *in vitro* systems. One of the first attempts was to select aptamers to components involved in clogging of the blood. During the last decade numerous publications of aptamers directed to thrombin and other proteins in the coagulase cascade have been reported. It is thus not surprising that we have found several such aptamers in clinical trials.

Aptamers have also been shown to inhibit targets such as enzymes and signal substances and to reduce the development of tumours, age-related loss of sight, cardiovascular and renal diseases. Furthermore, receptors to such molecules are attractive candidates for aptamer selection. Anti-cancer aptamers are other examples of successful drug developments, although none of them so far are ready for marketing. Abnormal forms of proteins and peptides, which have a normal function in the human body, are also examples of targets for aptamer selection. In addition, aptamers have been selected to autoantibodies, which are involved in many diseases.

There is a need for developing new therapeutical means for infectious diseases, mainly those caused by viruses and parasites, and aptamers directed to several such organisms have been described. Aptamers have also been outlined for targeted

delivery of therapeutic agents such as radioactive and toxic substances. The small size of the aptamers makes them suitable for such delivery as they easily penetrate tissues.

Aptamers are adjustable for the desired application, i.e. modified bases may be integrated, in order to increase the *in vivo* stability. Several of the reviewed publications have shown that a considerable resistance to human serum (prolonged half-lives) is obtained after modifications of the aptamer sequences. As a consequence, the doses required for treatment are reduced, the risk for side-effects is decreased and there is also a financial benefit.

The present review of the aptamer research field makes it clear that many of the aptamers have so far only been evaluated in animal model systems. There are very few reports of clinical trials with therapeutic aptamers available. These aptamers are directed to crucial proteins in cancer and eye diseases. On the contrary, we have observed that there are several hundred patents and patents applications on aptamers and among these patents there are several promising candidates for therapy.

Even if there are no aptamer-based drugs commercially available, some aptamers are in a late stage of development and have entered clinical trials. The lack of commercial products is not unexpected, since it normally takes at least 8-10 years of developmental work before a new drug can be introduced.

In assessing the success of aptamers as therapeutic agents it is interesting to compare with the development of monoclonal antibodies as therapeutic agents. When monoclonal antibodies were first generated in 1975 by the hybridoma technology it was expected that their high specificity and unlimited supply would ensure that therapeutic products would soon follow. In fact, as is often the case with new technologies, expectations far exceeded reality and many of the difficulties in dealing with this new class of drugs were not appreciated. The first FDA[142]-approved monoclonal antibody for therapeutical use was introduced in 1986, 11 years later, and the second in 1994, 19 years later. In January 2004 there were 14 monoclonal antibodies on the market for therapeutical applications[143] such as inhibition of alloimmune and autoimmune reactivity, anti-tumor therapy, anti-platelet therapy and anti-viral therapy. Furthermore, there are 179 antibodies undergoing different phases of clinical trials for a variety of conditions according to CenterWatch Drugs in Clinical Trials Database.[144] Bearing all this in mind, the first aptamer-based drug should not be expected on the market until the coming years.

The commercial application of aptamers for analytical and diagnostic purposes requires that aptamer-based techniques are as good as, or even better, than already existing commercially available techniques. Since the aptamer field still is in a developmental phase it is too early to predict what the future will hold in its hands. However, some of the developed assays and sensors show very promising performance characteristics. Especially, the photoaptamer-based protein arrays could be close to commercialisation. The company SomaLogic, Inc., developing the technique, has announced that they will have products out on the market in the near future and they also entered into collaboration with Beckman Coulter, Inc. to combine the photoaptamer technology with the BioMek®liquid handling workstation.

[142] FDA, Food and Drug Administration (USA)
[143] Medarex web-page, www.medarex.com
[144] www.centerwatch.com

In addition, aptamers for treatment of solid tumours are attractive candidates as approved drugs in the future.

In conclusion, the development of aptamers for therapeutical, analytical and diagnostic use is an expanding area with a promising future.

Acknowledgements

We are thankful to Gudrun Cassel and Calle Nilsson for valuable discussions and suggestions. We also thank Carin Stenlund for database searches and literature, Marianne Olofsson for corrections of the English text and Marie Lindgren for help with the lay-out of the manuscript.

Appendix 1. Example of aptamers for which structural data are available in the Protein Data Bank[145]

Target	Aptamer	Structure	Exp. Method	Deposition date	Accession number
AMP	RNA	Complex (AMP)	NMR	19/06/1997	1AM0
ATP	DNA	Complex (AMP)	NMR	09/10/1997	1AW4
	RNA	Complex (AMP)	NMR	17/07/1996	1RAW
FMN	RNA	Complex	NMR	04/12/1995	1FMN
Argininamide	DNA	Complex	NMR	02/11/1999	1DB6
	DNA	Complex	NMR	19/08/1998	2ARG
Arginine	RNA	Complex	NMR	28/03/1996	1KOC
Malachite Green	RNA	Complex (tetramethylrosamine)	X-ray diffr.	19/05/2000	1F1T
Citrulline	RNA	Complex	NMR	28/03/1996	1KOD
Vitamin B12	RNA	Complex	X-ray diffr.	12/11/1999	1DDY
(Cobalamin)	RNA	Complex	X-ray diffr.	12/04/2000	1ET4
Theofylline	RNA	Complex	NMR	20/03/1997	1EHT
	RNA	Complex	NMR	21/10/2002	1O15
Biotin	RNA	Aptamer	X-ray diffr.	23/05/2000	1F27
Neomycin B	RNA	Complex	NMR	15/03/1999	1NEM
Streptomycin	RNA	Aptamer	X-ray diffr.	29/01/2003	1NTA
	RNA	Complex	X-ray diffr.	29/01/2003	1NTB
Tobramycin	RNA	Complex	NMR	17/06/1998	2TOB
	RNA	Complex	NMR	12/12/1996	1TOB
Rex peptide, HTLV-1	RNA	Complex	NMR	05/05/2000	1EXY
Rev peptide, HIV-1	RNA	Complex	NMR	05711/1996	1ULL
	RNA	Complex	NMR	02/08/1999	484D
Tat protein, HIV	RNA	Complex (argininamide)	NMR	03712/2002	1NBK
Coat protein, MS2	RNA	Complex	X-ray diffr.	21/08/2001	1GKV
	RNA	Complex	X-ray diffr.	21/08/2001	1GKW
	RNA	Complex	X-ray diffr.	09/02/2001	1H8J
	RNA	Complex	X-ray diffr.	17/11/2000	1HDW
	RNA	Complex	X-ray diffr.	17/11/2000	1HE0
	RNA	Complex	X-ray diffr.	20711/2000	1HE6
NF-κB (p50)	RNA	Complex	X-ray diffr.	03/03/2003	1OOA
Thrombin	DNA	Aptamer	NMR	15/11/1993	148D
	DNA	Aptamer	NMR	24/07/1999	1C32
	DNA	Aptamer	NMR	24/07/1999	1C34
	DNA	Aptamer	NMR	25/07/1999	1C35
	DNA	Aptamer	NMR	25/07/1999	1C38
	DNA	Complex	X-ray diffr.	03/10/1995	1HAO
	DNA	Complex	X-ray diffr.	03/10/1995	1HAP
	DNA	Complex	X-ray diffr.	27/05/1993	1HUT
	DNA	Aptamer	NMR	05711/2003	1RDE
	DNA	Aptamer	NMR	11/04/1996	1QDF
Nucleolin	RNA	Aptamer	NMR	05/04/2001	1IE2

[145] Berman, H.M., *et al*. 2000. The Protein Data Bank. Nucleic Acids Res. 28: 235-242, URL<http://www.rscb.org/pdb>.

Appendix 2. Examples of recent (year 2003-2004) aptamer-related patents in the USPO[146] and EPO[147] patent databases

Titel	Area	Inventors	Assignee/ Applicant	Patent No
SELECTION/ISOLATION OF APTAMERS				
Method for the automated generation of nucleic acid ligands	A method and device for performing automated SELEX.	Gold, L., *et al*.	SomaLogic, Inc. (US)	US6,569,620
Conditional-SELEX	Methods for producing nucleic acid ligands that generate a signal, or cause a decrease in the level of a signal, in the presence of a target molecule or an environmental stimulus.	Smith, J.D. and Gold, L.	SomaLogic, Inc. (US)	US6,506,887
Aptamers containing sequences of nucleic acids or nucleic acid analogues bound homologously, or in novel complexes	Aptamers comprising at least two nucleobase-containing sequences, which are parallel or antiparallel to each other, and bound by Watson-Crick or homologous binding preferences. A rapid and sensitive and safe method for assaying binding between aptamers and target (exemplified by c-JUN, SpI and Oct-1).	Erikson, G.H. and Daksis, I.	Ingeneus Corp. (BB)	WO03027319
APTAMERS DIRECTED TO DIFFERENT TARGETS				
High affinity nucleic acid ligands of complement system proteins	Methods for the identification and preparation of high-affinity nucleic acid ligands to complement system proteins (C1q, C3 and C5).	Biesecker, G. and Gold, L	Gilead Sciences, Inc. (US)	US6,566,343
2'-fluoropyrimidine anti-calf intestinal phosphatase nucleic acid ligands	Methods for the identification and preparation of nucleic acid ligands to calf instestinal phosphatase	Drolet, B. and Gold, L.	Gilead Sciences, Inc. (US)	US6,673,553
Aptamer specifically adsorbable to Verotoxin I and method for obtaining the same	Method for obtaining an aptamer which specifically recognize and adsorb Verotoxin I produced by enterohaemorrhagic *Escherichia coli* .	Okada, K., *et al.*	Nitto Denko Corp. (JP)	JP2003079370
Pain receptor gene and protein	A novel pain receptor protein. The innovation includes an aptamer directed against the receptor.	Kubish, C. and Ramirez, A.	Rheinische, F.-M. Univ Bonn (DE)	WO03091281
DIAGNOSIS AND ANALYSIS				
Tenascin-C nucleic acids ligands	Methods for identification and preparation of nucleic acid ligands to tenascin-C. Methods for detecting the presence of a disease condition in a biological tissue in which tenascin-C is expressed.	Hicke, B., *et al.*	Gilead Sciences, Inc. (US)	US6,596,491

[146] US Patent & Trademark Office, URL<http://www.uspto.gov/>.
[147] European Patent Office, URL<http://ep.espacenet.com/>.

Titel	Area	Inventors	Assignee/ Applicant	Patent No
Signalling aptamer complexes	Aptamer based fluorescent reporters that function based on a switch from DNA/DNA duplex conformation to DNA/target confirmation.	Li, Y. and Nutiu, R.	Univ McMaster (US)	WO03062422
Deoxyribozymes	Methods for the detection and isolation of nucleic acid enzymes which possess desired characteristics. A fluorescence-generating RNA-cleaving DNA enzyme system that links chemical catalysis with real-time fluorescence signaling capability.	Li, Y., *et al.*	Univ McMaster (US)	WO03068963
Displacement assay for detection of small molecules	Complex of an anti-cocaine aptamer and the dye diethylthiotricarbocyanine as a sensor for cocaine in the concentration range of 2-5000 μM.	Landry, D.W. and Stojanovic, M.N.	Univ Columbia (US)	WO03089902
Determining non-nucleic acid molecule binding to target by competition with nucleic acid ligand	A high throughput competitive binding assay to determine whether a non-nucleic acid molecule from a library binds to a target. Detecting displacement of a nucleic acid ligand from a complex determines the binding of the non-nucleic acid molecule to the target.	Janjic, N. and Gold, L.	Gilead Sciences, Inc. (US)	US6,670,132
Nucleic acid ligand diagnostic biochip	A diagnostic biochip comprising a solid support to which one or more specific nucleic acid ligands is attached. Each ligand binds specifically and avidly to a particular target molecule contained within a test mixture. Methods for preparing biochips. Methods for the use of biochips in diagnosis of medical condition and quantitative detection of a target molecule in any chemically complex mixture.	Gold, L. e*t al.*	SomaLogic, Inc. (US)	US6,544,776 US6,503,715
Phosphoromonothioate and phosphorodithioate oligonucleotide aptamer chip for functional proteomics	An apparatus and method for monitoring biological interaction. The apparatus includes a substrate, a modified nucleotide aptamer attached to the substrate, a target molecule or portion thereof, wherein the interaction between aptamer and target molecule is detected.	Luxon, B. *et al.*	Univ Texas, (US)	WO03050290
Homogeneous detection of a target through nucleic acid-ligand-ligand beacon interaction	An assay, the ligand beacon assay, which utilizes molecular beacons as the reporter and nucleic acid ligands as the sensor for detection of target molecules in a test mixture. The assay specifically detects the molecular target that binds the nucleic acid ligand with high affinity and specificity.	Jayasena, S. and Gold, L.	Gilead Sciences, Inc. (US)	US6,531,286

Titel	Area	Inventors	Assignee/ Applicant	Patent No
THERAPY				
Aptamers and antiaptamers	An aptamer comprising a circular oligonucleotide with one to four thrombin binding regions.	King, G.C.	Unisearch Ltd (AU)	WO03002592
Aptamer constructs	RNA oligonucleotides having an aptamer flanked by two self-cleaving ribozymes. Methods of inhibiting replication of a virus in a cell, methods of treating an organism with an aptamer, methods of determining whether a test aptamer is effective in inhibiting the function of a target in a cell, and methods of expressing an RNA aptamer in a cell are provided.	Prasad, V. and Joshi, P.	Albert Einstein College of Medicine, Yeshiva Univ (US)	WO03102146
Aptamer-toxin molecules and methods for using the same	Materials and methods for preparation of therapeutic conjugates for the treatment of proliferative diseases. The conjugates comprise a targeting molecule (aptamer) conjugated to a therapeutic moiety with cytotoxic effect.	Kurz, M. and Stanton, M.	Archemix, Inc. (US)	WO03106659
APTAMERS AND DRUG-DELIVERY				
Nucleotide-based prodrug	Nucleotide-based prodrugs and their drug-delivery applications. The invention comprises a drug component covalently attached to one or more nucleotide components. Release and activation of the drug component arises from hydrolysis of the covalent bond. The nucleotide component provides means of targeting and/or anchoring to the desired tissue compartment and/or a mechanism of sustained release.	Warren, S.	Gilead Sciences, Inc. (US)	US6,610,841
Platelet derived growth factor (PDGF) nucleic acid ligand complexes	Method for preparing a complex comprised of a PDGF nucleic acid ligand and a non-immunogenic, high molecular weight compound or lipophilic compound.	Janjic, N. and Gold, L.	Gilead Sciences, Inc. (US)	US6,582,918
Ocular iontophoretic device and method for inhibiting vascular endothelial growth factor (VEGF) using the same	A device for delivering an aptamer to an affected area of a living being's eye for inhibiting VEGF function therein.	Lloyd, L.B. *et al.*	Iomed, Inc. (US)	US6,579,276
Ocular drug delivery systems and use thereof	A microspore formulation for the sustained delivery of an aptamer (i.e. an anti-VEGF aptamer) to a preselected locus (i.e. scleral surface of an eye) in a mammal.	Adamis, A. and Carrasquillo, K.G.	Massachusetts Eye and Ear Infirmary (US)	WO03092665

Appendix 3. Detection limits of some aptamer-based analytical methods

Target	Method	Sensitivity	Linear range	Sample volume	Time for analysis	Ref
PDGF	Proximity-dependent DNA ligation assay, utilizing PCR	40 x 10^{-15} M 40 x 10^{-21} mol 24,000 PDGF-BB molecules, 1 fg protein	>1,000-fold concentration range	1 or 3.4 µL		93
	Fluorescence anisotropy sensor Fluorophore-labelled aptamer	220 x 10^{-12} M	0-100 nM		~22 sec	92
Alpha-Thrombin	Proximity-dependent DNA ligation assay	~10^{-19} mol				93
	Affinity probe capillary electrophoresis Fluorophore-labelled aptamer	40 x 10^{-9} M 37 x 10^{-21} mol	Dynamic range of 10^5	30 µL		81
	Biosensor Fluorescently-labelled aptamer Changes in evanescent-wave-induced fluorescence anisotropy	5 x 10^{-9} M 700 x 10^{-21} mol	3 orders of magnitude	140 pL	< 10 min	97
	Biosensor, fiber-optic microarray system Competetive assay utilizing fluorescein labelled thrombin and aptamer-coated beads	1 x 10^{-9} M	nM to low µM	10 µL	~15 min	98
Human IgE	Quartz crystal biosensor	500 x 10^{-12} M 100 µg/L	Linear up to 10 mg/L			96
	Affinity probe capillary electrophoresis Fluorophore-labelled aptamer	46 x 10^{-12} M	Linear dynamic range of 10^5	30 µL	60 sec	81
$bFGF_{(155)}$	Photo-SELEX	3.2 x 10^{-12} M 58 pg/ml		20 µL		102
RT, HIV-1	Affinity capillary electrophoresis Fluorescently labelled aptamer	~10 x 10^{-9} M	Linear up to 50 nM (6µg/ml)		< 5 min	87
RT ARM, HIV-1	Aptazyme (allosteric ribozyme) ligase array	60 x 10^{-9} M				148
Cocaine	Fluorescent sensor Folding aptamer labelled with fluorophore and quencher	10 x 10^{-6} M	10 – 4000 µM			99
Lysozyme	Aptazyme (allosteric ribozyme) ligase array	400 x 10^{-12} M 82 x 10^{-15} mol				148
Theofylline	Fluorescing molecular switch	10 x 10^{-6} M	0,01-2 mM			149
FMN	Aptazyme (allosteric ribozyme) ligase array	370 x 10^{-9} M				148

[148] Hesselberth, J.R., *et a*l. 2003. Simultaneous detection of diverse analytes with an aptazyme ligase array. Anal. Biochem. 312:106-112.
[149] Frauendorf, C., and Jäschke, A. 2001. Detection of small organic analytes by fluorescing molecular switches. Bioorg. Med. Chem. 9:2521-2524.