Artificial Aptamers

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Nucleic Acid and Peptide Aptamers: Fundamentals and Bioanalytical Aspects

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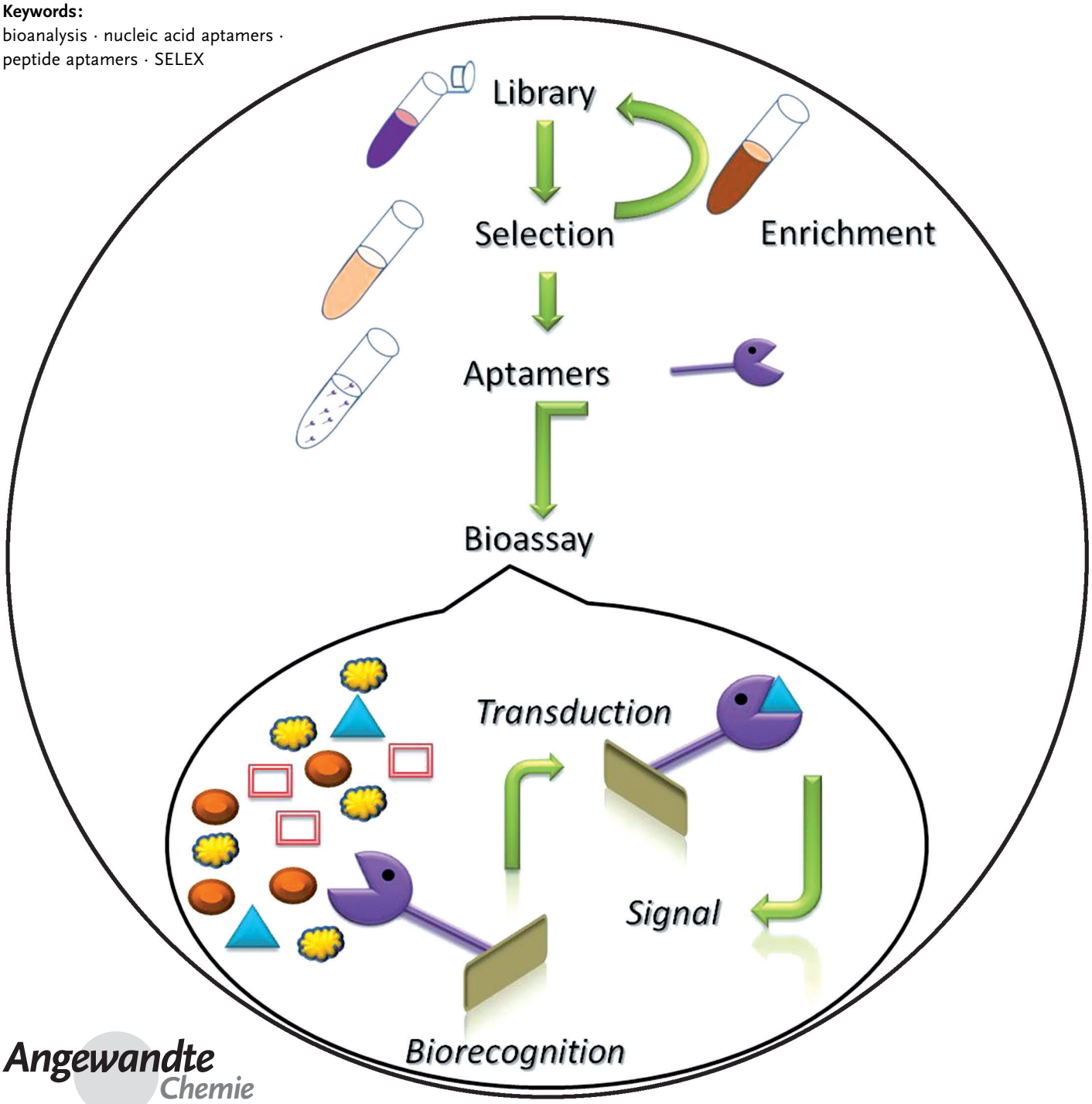

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In recent years new nucleic acid and protein-based combinatorial molecules have attracted the attention of researchers working in various areas of science, ranging from medicine to analytical chemistry. These molecules, called aptamers, have been proposed as alternatives to antibodies in many different applications. The aim of this Review is to illustrate the peculiarities of these combinatorial molecules which have initially been explored for their importance in molecular medicine, but have enormous potential in other biotechnological fields historically dominated by antibodies, such as bioassays. A description of these molecules is given, and the methods for their selection and production are also summarized. Moreover, critical aspects related to these molecules are discussed.

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1. Introduction

Nowadays, the scientific application of antibodies ranges from basic studies to applied medicine. Antibody-based products have been approved as biopharmaceuticals for the treatment of cancer, chronic inflammatory diseases, transplantation, infectious diseases, and cardiovascular medicine.[1] A few hundred antibodies at least are drug candidates under clinical development.[1] Moreover, antibody-based bioassays are routinely used in clinical, environmental, and food analysis. Most of these bioassays are based on the immunoglobulin G (IgG) molecule. IgG is a 150 kDa molecular mass protein composed of four polypeptide chains with disulfide bonds that are essential for its stability. In addition, these proteins possess a complex glycosylation pattern. These characteristics lead to a comparatively difficult and expensive production process, exacerbated by the use of animals. Significant progress has been made in developing stable recombinant antibody fragment libraries; however, valuable alternatives are still required.[2]

Recently, attention has turned toward affinity molecules produced by evolutionary molecular biology approaches[3–6] (Figure 1). This means that a combinatorial library is constructed, and improved variants are identified through a selection process. The selection process is performed in vitro, thereby allowing selection itself to be most conveniently controlled, thus retaining control of the characteristics of the identified affinity molecule.

The aim of this Review is to illustrate the peculiarities and the applications in bioassays of the two major classes of affinity molecules produced by evolutionary approaches, namely nucleic acid aptamers and combinatorial non-immunoglobulin proteins (termed here, for convenience, simply peptide aptamers).

To better clarify the terminology, the name "aptamer", derived from the Latin expression "aptus" (to fit) and the Greek word "meros" (part), was first used in 1990 by Ellington and Szostak to describe RNA molecules that bind to a small organic dye.[7] Since then, short strands of DNA or RNA that adopt specific three-dimensional conformations and that are selected for targeting distinct molecules have been termed nucleic acid aptamers. The development of

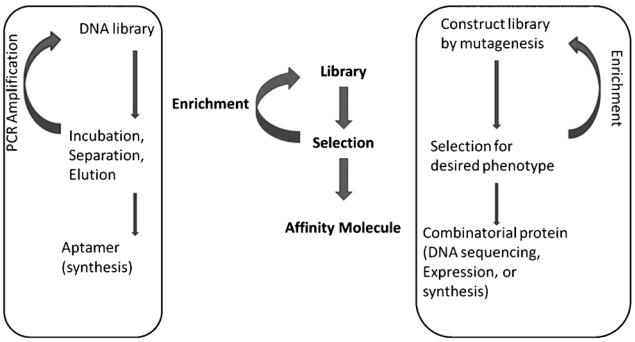

Figure 1. Scheme of the evolutionary approach. The nucleic acid aptamers (left box) are produced by incubation of the nucleic acid library (DNA library) with a target molecule of choice, separation of bound from free nucleic acid species, elution of bound nucleic acid species, and amplification of eluted nucleic acid species. In the case of the combinatorial proteins (right box), a combinatorial library of mutated genes is synthesized. Improved variants are identified through a selection process. Further improvements may be gained from iterative cycles of mutation and selection. Finally, clones are characterized by DNA sequencing to identify beneficial mutations. The general approach is described in the center.

artificial combinatorial proteins as alternatives to antibodies (consisting of a variable peptide sequence inserted within a constant scaffold protein) was reported some years later. Among others, in 1995 the research group of Nygren[8] reported the construction of a combinatorial library of a α-helical bacterial receptor. A year later, Colas et al.[9] reported the development of a thioredoxin A (TrxA) based affinity protein. These authors defined this TrxA-based molecule a "peptide aptamer" by analogy to nucleic acid aptamers.

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Different combinatorial non-immunoglobulin proteins have been reported in the literature and all of these molecules are identified by different names. To have a rational approach, we decided to review the vast number of publications in this field under a generic term. We have chosen, for convenience, the term peptide aptamers. In our opinion, the use of the term "aptamer" will help the reader to focus on the final goal of the Review: a discussion on affinity molecules (nucleic acids as well as proteins) obtained from an evolutionary approach, and which represent interesting alternatives to antibodies.

2. Nucleic Acid Aptamers

2.1. Definition and Description

Nucleic acid aptamers are short, single-stranded DNA or RNA oligonucleotides which adopt stable three-dimensional sequence-dependent structures. This intrinsic property makes them efficient binding molecules, capable of binding to molecular targets, ranging from small ions (e.g. Zn^{2+},[10] 56 Da) and small organic compounds (e.g. organic dyes,[11] neutral disaccharides,[12] and aminoglycoside antibiotics[13]) to large molecules such as glycoproteins (such as CD4[14]) or even a complex target (e.g. living cells[15]). The functionality of nucleic acid aptamers is based on their stable three-dimensional structure, which is dependent on the primary sequence, the length of the nucleic acid molecule, and the environmental conditions. Aptamers can vary in size from 25 to 90 bases,[5,6] and their typical structural motifs are stems,[16] internal loops, purine-rich bulges, hairpin structures, tetraloops,[17] pseudoknots,[18,19] kissing complexes,[20,21] or G-quadruplex structures[22–24] (namely tertiary structures similar to those observed with RNA and DNA[25]). Some examples are shown in Figure 2. In the presence of the target, most of the aptamers undergo adaptive conformational changes and their three-dimensional folding creates a specific binding site for the target. The intermolecular interactions between the aptamer and the target are characterized by a combination of complementarity in shape, stacking interactions between aromatic compounds and the nucleobases of the aptamers, electrostatic interactions between charged groups, and hydrogen bonds.[26]

Nucleic acid aptamers bind to their targets with high specificity, so that differentiation on the basis of minor structural differences between targets and their related molecules can be obtained. For example, theophylline and its analogues caffeine and theobromine have similar chemical structures. Caffeine differs from theophylline by a single methyl group, while theobromine is actually an isomer of theophylline with the methyl group in a different position. The anti-theophylline RNA aptamers displayed high levels of molecular discrimination against both analogues, and it has been proven that the binding affinity of one RNA aptamer to theophylline is 10000-fold higher than to caffeine.[27] Similarly, RNA aptamers selected for L-arginine can enantioselectively bind to this target with 12000-fold higher affinity than to D-arginine.[28]

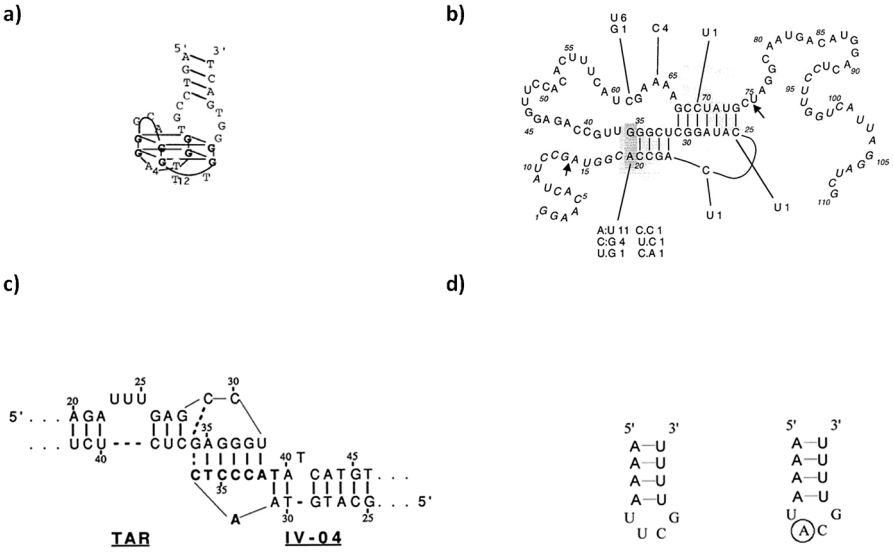

Figure 2. Examples of structural motifs of nucleic acid aptamers: a) a thrombin-binding aptamer folded as a G-quadruplex structure;[24] b) a sequence of the major biotin aptamer clone folded as a pseudoknot;[19] c) the IV-04 aptamer against transactivation-responsive (TAR) RNA-forming RNA-DNA kissing complex;[20] d) sequences of aptamers folded as a stem loop.[16]

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There is a large variation in the affinity both among aptamers for small molecules and aptamers for proteins. The properties of small molecules which promote the best recognition, and lead to affinities in the micromolar range,[29,30] are planarity or the presence of positively charged groups and hydrogen-bond donors or acceptors,[31] even if aptamers for molecules with a hydrophobic character have been selected.[32,33]

Aptamers for proteins generally exhibit higher affinities, because of the presence of larger complex areas with structures rich in hydrogen-bond donors and acceptors. Affinities in the nanomolar and subnanomolar range have been measured for aptamers against different proteins, such as thrombin (25 nM),[22] nucleocapsid protein (2 nM),[34] and platelet-derived growth factor (PDGF; 0.1 nM).[35]

2.2. The Selection Process and its Evolution

The aptamer isolation process, called SELEX (systematic evolution of ligands by exponential enrichment), was first reported in 1990 almost simultaneously by the research groups of Ellington[7] and Tuerk.[36] This technique essentially consists of the repeated binding, selection, and amplification of aptamers from the initial library until one (or more) aptamers displaying the desired characteristics have been isolated.[7,36] The SELEX process has been extensively reviewed[37–43] and several modifications of the process have been introduced. A brief description of the method and its latest variants will be described below.

The initial and very important step of the SELEX process is the choice and synthesis of the library. Part of the enormous potential of aptamers lies in the fact that libraries with vast numbers of potential ligands can be created and enriched within a few days. Typically, aptamer libraries consist of 10^{13}–10^{18} random oligonucleotide sequences[44,45] and this is even more impressive when compared to conventional libraries of potential drugs which consist generally of no more than 10^6 different molecules and may take months to screen.

When creating a library, however, several factors need to be taken into account, such as the complexity of the library and the chemistry of the nucleotides. In particular, the chemistry of the nucleotides plays a central role in regard to the stability of the aptamer towards degradation. It can also influence the affinity and the specificity of the selected aptamers towards their targets since many of the nucleotides at the heart of aptamers determine the overall structure of the binding site.[41,46] In fact, modification of nucleotide bases has been the most commonly used method to avoid the susceptibility of single-stranded oligonucleotides to enzymatic or chemical cleavage. Modification of pyrimidines at the 5′-position with I, Br, Cl, NH_3, and N_3 and at the 2′-position with NH_2, F, and OCH, for example, has been described.[39] The modification of the phosphodiester backbone, for example, through the use of α-thio-substituted deoxynucleotide triphosphates, was shown to be a useful method, more successful with DNA aptamers than RNA aptamers.[47] An alternative is represented by the generation of enantiomeric aptamers, known as "spiegelmers" (from the German word for "mirror"). This technique consists of creating a mirror image of the target and selecting an aptamer for this mirror image. A stereoisomer of the selected aptamer is then created (for example, the spiegelmer), which will be specific for the target but will not be susceptible to normal enzymatic degradation because of the substitution of the natural D-ribose with L-ribose.[48–50]

The other factor influencing the design of the library is the choice of the constant region. The random aptamer sequence has to be flanked by constant sequences at 5′ and 3′. These sequences are usually 20–25 base pairs in length and provide hybridization sites during a number of steps of the SELEX process. The 3′-flanking sequence generally acts as an attachment site for the reverse transcriptase primer, while the 5′-flanking sequence acts as the attachment site for the PCR primers during the amplification step of the SELEX protocol. The design of the constant region for the SELEX procedure is even more important than for normal PCR, given that a complete SELEX process may include up to 50 cycles of PCR. Any artifacts would thus be drastically amplified in the enriched library. After a suitable aptamer library has been prepared, it can undergo the SELEX protocol, which starts from the designed double-stranded DNA library, which either needs to be transcribed (for RNA selection) or strand-separated (for single-stranded DNA selection) to be in a suitable form for selection. In the following step, the target and the library are brought together under favorable binding conditions, where the sequences with the highest affinity will bind to the target. These sequences are then partitioned from those with lower affinity. This step can be performed by attaching the aptamers to a solid-phase support, such as sepharose, and specifically eluting the desired aptamers after binding has taken place.[5,51] Alternatively, the aptamer and target could be allowed to interact freely in solution, after which the target–aptamer complex could be recovered by filtration through nitrocellulose.[38,52] This method is commonly used, although it is important to note that it is only applicable when the target molecule is a protein. A negative selection step is also frequently used at this stage, in which the aptamers are passed over a cellulose filter in the absence of the target or over the matrix on which the target is immobilized (negative SELEX). This is to eliminate aptamers that bind to the filter or to the matrix in a target-independent manner.[28] Counterselection is also sometimes used, where aptamers that bind structures similar to that of the target are removed.[53] The high affinity sequences are then amplified by

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reverse-transcription PCR (RT-PCR; for RNA aptamers) or by PCR (for DNA aptamers) to create a new aptamer library enriched with the aptamers of high affinity. The entire process is then repeated, thereby resulting in fewer and fewer unique sequences with higher and higher affinity to the target being retained. The binding conditions for the aptamer and the target are generally made more stringent during each round of selection to increase the selective pressure on the remaining aptamers. A complete SELEX process (between 8 and 15 cycles) will yield several individual sequences which are analyzed and combined in several classes according to their homology to each other. Further investigation comprises analysis of conserved motifs of the aptamer sequence and elucidation of the minimal aptamer dimensions sufficient for interaction with the target.[41]

Several modifications of the SELEX procedure[54] (Table 1)[55–85] have been introduced to improve the aptamer selectivity (blended, counter, negative, and subtractive SELEX), to reduce the time for selection (automated SELEX), or to improve the efficiency of the partitioning step (CE SELEX and non-SELEX). Moreover, particular SELEX protocols have been created to select aptamers towards particular targets, such as complex target SELEX or cell SELEX, or for proteomics studies and biomarker discovery.[86] All these different SELEX procedures, which aim to direct the selection to aptamers with desired features, have been cited in this Review to illustrate an important difference between aptamers and antibodies. In contrast to the plethora of possible modifications of the SELEX conditions, the classical production of antibodies with animal immunization is difficult to influence, and the use of physiological conditions are the decisive factor.

Particular and innovative SELEX procedures have been recently reported, such as AFM-SELEX.[87] Atomic force microscopy (AFM) was used to obtain aptamers with strong affinity for the target: selection was completed after only three rounds, and many of the obtained aptamers had a higher affinity to the target, thrombin, than those selected by conventional SELEX. A recent study presents an automatic, magnetic bead based microfluidic system which integrates a random ssDNA extraction device and an on-chip nucleic acid amplification device (micro-PCR) for the fast screening of aptamers.[88] The entire process was performed automatically on a single chip within a shorter period of time than other SELEX protocols and with lower amounts of samples and reagents.

Moreover, an alternative microfluidic SELEX based on micromagnetic separation was also published recently.[89] The micromagnetic separation chip incorporates microfabricated ferromagnetic structures to trap aptamers bound to magnetic beads, and demonstrated a high efficiency in the partitioning step. As a proof of principle, an aptamer selection for streptavidin was performed, and after only three rounds highly affinity aptamers were generated with dissociation constants ranging from 25 to 65 nM.

3. "Peptide Aptamers"

3.1. Definition and Description

Strictly following the definition coined by Colas et al. in 1996,[9] peptide aptamers are combinatorial protein molecules in which a variable peptide sequence with affinity for a given target protein is displayed on an inert, constant scaffold protein.[9,90–95] They are extremely simple molecules, selected from combinatorial libraries on the basis of their affinity to the target protein or small molecule, and expressed in bacterial cells, such as *E. coli*. Both termini of the variable sequence are fused to the inert scaffold, thus peptide aptamers are doubly constrained. This double constraint distinguishes peptide aptamers from other artificial combinatorial protein molecules, which often consist of random peptidic sequences fused terminally to a carrier protein or another macromolecule. Actually, the term does not comprise other types of double-constrained combinatorial proteins that are more complex than peptide aptamers because target-binding surfaces consist of noncontiguous peptidic sequences disseminated over several secondary structural elements or across several variable loops,[90,91] as depicted in Figure 3.

However, these double-constrained combinatorial proteins have similar characteristics and applications as peptide aptamers. In particular, all of them show molecular recognition properties, in a manner similar to antibodies, but with improved characteristics, such as small size, high stability, high solubility, high yield bacterial expression, possibility of chemical synthesis, rapid folding properties, and in some cases, such as in the affibody molecules (affinity molecules based on the protein A scaffold, see Table 2), absence of disulfide bonds and of free cysteine residues. As reported in Ref. [96], which highlighted the characteristics of the affibody molecules, the high stability in the absence of disulfide bonds is an important advantage, which facilitates high yields in bacterial expression and enables intracellular applications. Moreover, the absence of intramolecular cysteine residues gives the possibility of introducing a unique C-terminal cysteine residue for labeling or other chemical modifications.

The final shape of these artificial constrained combinatorial proteins will be determined both by the amino acid composition and sequence of the peptide as well as by the primary sequence and tertiary structure of the scaffold protein.[94]

Importantly, the binding affinity of these artificial proteins is greatly increased by the constraint applied by the scaffold, and this is the main advantage associated with the use of conformationally constrained peptides versus unstructured linear peptides.[97,98]

3.2. Selection

Clearly, the selection of peptide aptamers is, from a technical point of view, completely different from that used for nucleic acid aptamers. However, the basic principles are similar: the use of combinatorial strategies to generate diversity and create a pool of different candidates (the

Table 1: Different modifications of the SELEX process.

Name	SELEX modification	Aim	Ref.
automated SELEX	use of automated systems for the selection procedure	reduction of time needed for the selection	[55, 56]
blended SELEX	use of small ligands which can direct the sequence to a specific region of the target	selection of aptamers towards a specific epitope	[57, 58]
capillary electrophoresis SELEX	use of capillary electrophoresis (CE) for sequence partitioning	improvement of the separation process between sequences bound to the target and the other sequences	[59–61]
cell SELEX	use of whole living cells as target	selection of aptamers towards whole living cells	[62, 63]
chimeric SELEX	use of combined populations (e.g. fusion of already selected sequences)	selection of dual-function aptamers	[64]
complex target SELEX	use of membrane preparations or cells as targets in the selection process	selection of aptamers against cell-surface proteins	[65]
conditional SELEX	use of a regulator molecule during selection	selection of aptamers whose binding to the target molecule can be regulated	[66]
counter SELEX	use of molecules similar to the target in the selection, to exclude those sequences binding to them	selection of highly specific aptamers	[27, 55]
covalent SELEX	use of nucleotides modified with groups that can be activated	selection of aptamers which can form covalent links to the target protein (photoaptamers)	[67, 68]
deconvolution SELEX	development of a secondary selection	partitioning of aptamer pools evolved against multiple targets	[69, 70]
facs SELEX	use of a fluorescence-activated cell-sorting device to simultaneously differentiate and separate binding from nonbinding subpopulations of cells	decrease in the number of false positives in cell-selection approaches	[71]
FluMag SELEX	use of fluorescent labels for DNA quantification and use of magnetic beads for target immobilization	use of very small amounts of target for the aptamer selection, rapid and efficient separation of bound and free molecules	[72]
genomic SELEX	library composed of fragmented genomic DNA	selection of natural sequences binding bioactive proteins	[73]
mirror-image SELEX	use of mirror analogues of natural nucleotides (L-ribose or L-deoxyribose)	selection of nuclease-resistant aptamers	[56]
mod-SELEX	SELEX on a library of oligonucleotides with chemical substitutions	production of stable (nuclease-resistant) aptamers and generation of aptamers with conformations and target-binding surfaces not accessible using DNA or RNA	[74]
multistage SELEX	use of fused members of already screened pools	elucidating mechanisms of allosteric interactions in aptamers	[75]
negative SELEX	use of a "negative" cycle of selection, by performing incubation only with the matrix used for the target immobilization	selection of highly specific aptamers	[76]
non-SELEX	elimination of the PCR step	improvement of the separation step	[77]
primer-free SELEX (in genomic SELEX)	primer-annealing sequences are removed from the genomic library before selection	prevention of artifacts arising from the presence of structures created from the base-pair formation between the fixed flanking sequences of the library and the central genomic-derived fragments.	[78]
SELEX-SAGE (serial analysis of gene expression) or high-throughput SELEX	part of the SAGE protocol is used to link together oligomers extracted from SELEX with longer DNA molecules, which can be efficiently sequenced	sequencing up to several thousand binding sequences	[79]
SOMAmer (slow off-rate modified aptamers)	selection of aptamers with rationally designed modified nucleotides	enlarging the range of targets for which aptamers can be selected and selection of high-quality binding aptamers to be used in highly multiplexed proteomics arrays	[80]
subtractive SELEX	use in the complex target SELEX of molecules similar to the target (i.e. cells) to exclude those sequences binding to them	improvement of aptamer selectivity	[81]
tailored SELEX	library with reduced or no fixed regions	minimization of the aptamer size	[82, 83]
TECS-SELEX (target expressed on cell surface SELEX)	instead of the purified protein, recombinant proteins displayed on the cell surface are directly used as the selection target	selection of aptamers with high specificity and affinity to any cell-surface protein, also when the purified protein target cannot be easily obtained	[84]
toggle SELEX	use of different targets in the selection	selection of aptamers which can bind to several related proteins	[85]

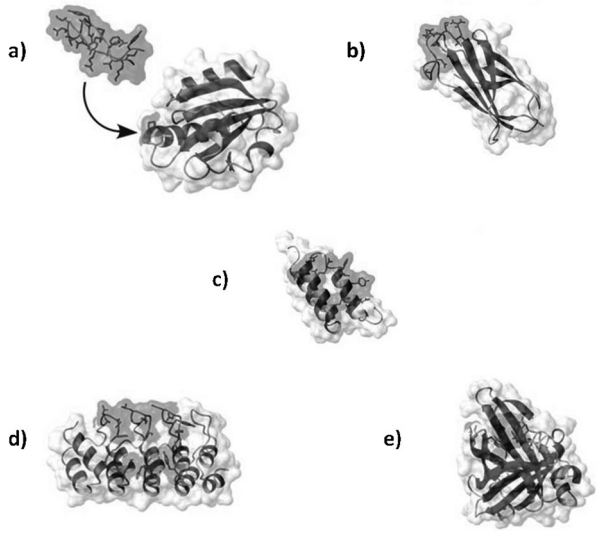

Figure 3. Representation of different protein scaffold principles for the engineering of artificial binding proteins. The scaffolds are diversified by a random peptide sequence inserted into the scaffold, usually at a loop, such as thioredoxin (a) or by engineering of noncontiguous specified positions disseminated over several secondary structural elements or across several variable loops (e.g., in loops such as fibronectin III (b), flat surfaces such as protein A (c), combinations of loops, and helices such as ankyrin repeat protein (d), or cavities such as lipocalin (e)). Target-binding variants of the resulting libraries are subsequently isolated by using selection or screening techniques. Reproduced from Ref. [3].

library), the selection (to find candidates with the best properties), and amplification.

Excellent reviews as well as book chapters dealing with the detailed technical aspects of combinatorial artificial protein selection technology are available[99,100] and, therefore, the methodology will be described here only briefly and only in a general form. In the next sections some aspects of the protein scaffold, of the library, and of selection technologies will be discussed.

3.2.1. ***Scaffold Selection and Library Design and Construction***

The first step in a combinatorial protein screen is to choose a peptide library. These libraries vary with respect to the choice of scaffold, peptide length, selection stringency, and the number of selectable markers. The scaffold is a protein framework that can carry altered amino acids or insertions, thereby giving protein variants with entirely novel functions and often new binding specificity. The choice of scaffold protein is mostly dependent on the intended use of the generated affinity ligands. However, the scaffold should preferably be relatively small, that is, composed of a single polypeptide chain, and with a highly stable architecture.[101] There are many protein scaffolds reported in the literature and they have been intensely reviewed in the past.[3,4,101–104] Only a selection of such scaffolds is presented here (see Table 2; the name coined for the resulting artificial proteins is also given), as exhaustive descriptions of them are reported elsewhere.[3,4,101–104]

The *E. coli* protein TrxA scaffold has been largely employed[91] for the development of peptide aptamers. TrxA is a robust enzyme with a short active site loop,[105] relatively small in size (approx. 12 kDa), with good stability and solubility, and with a well-known three-dimensional structure. Since the scaffold should be biologically inert, for the TrxA scaffold, the peptides are introduced into the loop within the biologically active center of the molecule, thereby destroying its catalytic activity. Recently, a protein scaffold, known as STM (stefin A triple mutant) and derived from the intracellular protease inhibitor stefin A, has been developed.[106] STM possesses three sites, distant from each other in the primary sequence of the protein, but adjacent in the folded

Table 2: Examples of scaffolds used for the generation of affinity ligands, method of selection, and commercial exploitation.

Name	Scaffold	Structural elements varied	Selection method	Reference	Commercial exploitation
peptide aptamer	thioredoxin A (TrxA)	1 loop	yeast two hybrid, phage display, mammalian cell system	[9,105]	– (up to 2007 by Aptanomics)
peptide aptamer	staphycoccus nuclease	1 loop	functional screening	[112]	–
peptide aptamer	human stefin A	3 sites	yeast two hybrid	[106]	–
peptide aptamer	green fluorescent protein	loop randomisation	visual screening	[111]	–
ligand-regulated peptide aptamers	FKBP-peptide-FRB-GST	trimeric complex		[108]	–
microbodies	cysteine-knot microproteins (knottins)	1 loop in two β strands	mRNA display	[119]	– (up to 2007 by Nascacell)
affibody	protein A	2 α helices	phage display	[96]	www.affibody.com www.abcam.com
anticalin	lipocalin	4 loops	phage display	[109]	www.pieris-ag.com Pieris libraries
Adnectin	fibronectin III	2–3 loops	phage display, mRNA display, yeast two hybrid	[110]	www.adnexustx.com PROfusion libraries
DARPin	ankirin	β turn and α helix	ribosome display	[113]	www.molecularpartners.com
Kunitz domain	APPI	single loop	phage display	[121]	www.dyax.com

protein and naturally used by stefin A to bind to target proteins.[106] Furthermore, the STM scaffold has been engineered to give reduced interactions between human proteins and the scaffold, thus reducing cross-reactivity in bioassays. The STM scaffold has been further mutated to introduce a single cysteine residue (STMcys+) so as to allow the oriented attachment of the scaffold to a solid surface through the exposed sulfhydryl group. Other variants of STM have recently been reported.[107] Miller et al. engineered a new ligand-regulated peptide (LiRP) system where the binding activity of intracellular peptides is controlled by a cell-permeable small molecule, such as rapamycin.[108] Other examples of scaffolds include anticalins modeled on lipocalin structures,[109] trinectins derived from a fibronectin III domain,[110] green fluorescent protein (GFP),[111] a catalytically inactive derivative of the staphylococcal nuclease (SNase),[112] the ankyrin repeat protein,[113] and "affibody molecules", which are engineered from the B domain of *Staphylococcus aureus* protein A.[114] This B domain is a relatively short cysteine-free peptide of 58 amino acids that is folded into a three-helical bundle structure and which has been engineered into a variant denoted the Z domain.[96] The Z domain retained its affinity for the Fc part of the antibody, while the weaker affinity for the Fab region was almost completely lost.[115–117] Cysteine-knot microproteins (also referred as knottins) are other interesting molecular scaffolds for the incorporation of foreign peptide sequences,[118–120] while the Kunitz domain is an example of a natural serine protease inhibitor that has been successfully utilized as a scaffold.[121]

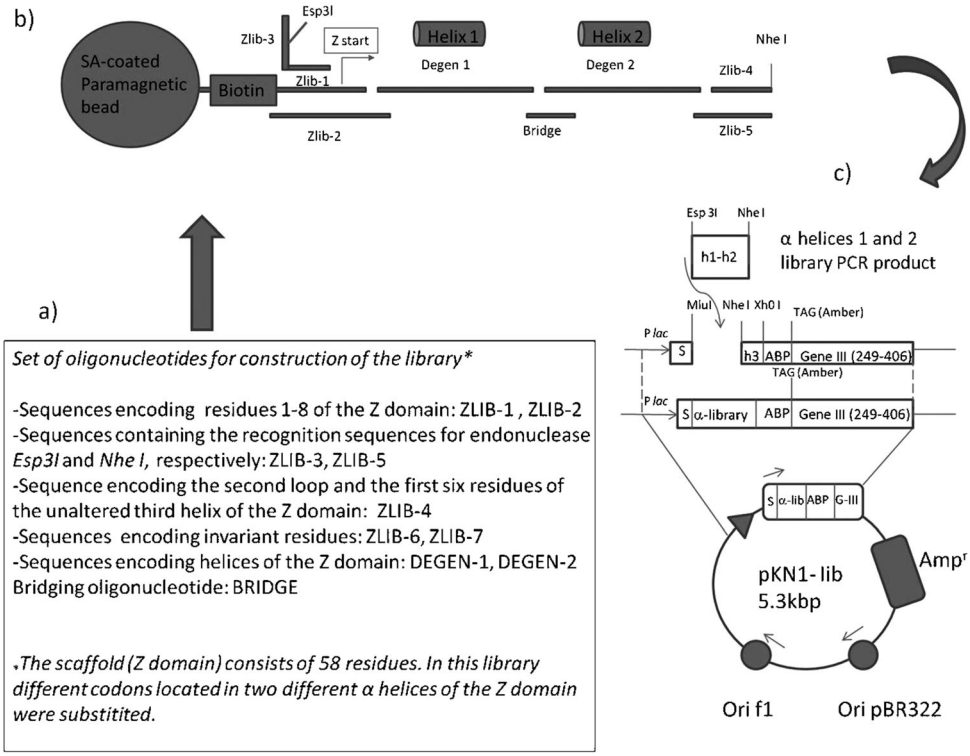

Figure 4. A schematic representation of library construction, adapted from Ref. [8]: a) Sequences of oligonucleotides used; b) the library was constructed using streptavidin-coated paramagnetic beads as a solid-phase anchor during assembly. Solid-phase-assisted assembly was initiated by binding of the 5′-biotinylated oligonucleotide pair ZLIB-l/ZLIB-2 to the beads. After washing the beads, the preformed construct DEGEN-l/BRIDGE/DEGEN-2 was added and ligated. Assembly was completed by the addition and ligation of the prehybridized oligonucleotides ZLIB-4/ZLIB-5. Prior to amplification by PCR, oligonucleotides ZLIB-2, BRIDGE, and ZLIB-5 were eluted with alkali. Oligonucleotides ZLIB-3 and ZLIB-5 were used as primers for PCR amplification. To obtain double-stranded DNA for cloning, the assembled and bead-immobilized single-stranded gene library encoding the two variegated helices of the Z domain was used as a template in PCR amplification. c) The library PCR product encoding the variegated helices 1 and 2 was subcloned into the phagemid vector pKN1, which contains the gene for residues 44–58 of the wild-type Z domain (essentially helix 3), followed by the gene for a 46 residue serum albumin binding region (ABP) derived from streptococcal protein G linked in-frame with a truncated version of the M13 phage coat protein III gene. The vector PKN1 was constructed in several steps as follows. A double-strand linker encoding the invariant residues 44–58 of the Z domain was formed from oligonucleotides ZLIB6 and ZLIB7. It was cloned as a MluI-XhoI fragment into phagemid pKP986, thereby resulting in pKN. Phagemid PK986 encodes the *E. coli* OmpA (S) leader peptide followed by residues 249–406 of M13 filamentous phage coat protein III under the control of the *E. coli lac* promoter.

Once the scaffold has been chosen, the combinatorial libraries of the scaffolds are produced. This is done at the DNA level by randomizing the codons at appropriate amino acid positions. In other words, the method used to construct the library of proteins consists of constructing a library of nucleic acid molecules (library of genes) from which the protein library can be translated. A wide range of techniques are now available for generating gene libraries,[122] but their description is beyond the scope of this Review. In Figure 4 an example of the construction of a combinatorial library of the α-helical Z-domain of protein A is reported (adapted from Ref. [8]).

Clearly, the combinatorial libraries of the scaffolds must be adapted for the particular selection system that will be employed (see Figure 4). This means that combinatorial libraries take the form of yeast two-hybrid libraries, as well as phage-display libraries, etc. Nowadays, libraries can be purchased from different companies, for example, from Clontech (for yeast two-hybrid selection, www.clontech.com), from Invitrogen ("FLITRX peptide library"), and others (see Table 2).

3.2.2. ***Selection Technologies***

Different selection methods are reported in the literature, and their use is strictly dependent on the intended use of the combinatorial proteins. Briefly, these methods can be classified as nondisplay systems, cell-dependent display systems, and cell-free display systems.[97] In the nondisplay systems, the target protein is coexpressed with the individual library members in vivo, and the selection is thus not dependent on an available target protein, but instead the target protein can be expressed in a correctly folded form by the host organism. Thus, in vivo techniques usually rely on the reconstitution of a protein activity when the binder interacts with the target (e.g., enzyme activity, fluorescence, or transcriptional activity). The yeast two-hybrid (Y2H) system is an example of in vivo technology.[123]

In this screening method a target protein is linked to a heterologous DNA binding domain (BD) and expressed as "bait" in a yeast test strain. Concomitantly, a library of different peptides, which are linked to a heterologous transcriptional activation domain (TAD), is expressed as "prey" (Figure 5a).

If a peptide binds to the target protein, a transcription factor is formed, in which the BD and activation domain (AD) are bridged by the interacting proteins. This transcription factor is then able to activate the promoter of a marker gene, which can be monitored by colorimetric enzymatic assays or by growth selection. This screening procedure results in the immediate availability of the binding molecule in virtually unlimited amounts. The vector, which encodes the binding molecule, can be isolated from the yeast test strain and the DNA sequence of its insert can be easily determined. This insert can then be introduced into suitable expression vectors for the synthesis of the binding molecules in bacteria or eukaryotic expression systems.

The protein-fragment complementation assay (PCA) or the mammalian cell screen are other examples of in vivo nondisplay techniques. PCA relies on the principle that the survival of cells simultaneously expressing complementary fragments of the enzyme murine dihydrofolate reductase (mDHFR) is dependent on the correct folding and interaction of these fragments.[124]

Phage display is an in vitro cell-dependent display technique invented by G. Smith in 1985.[125] In phage display, peptide or protein libraries are fused to the coat proteins of phages (mostly geneIII protein), which are displayed on the surface of the phage particle (Figure 5b). The phage is then incubated with the target molecule. After the selection, any unbound phage is washed away and the phage specifically binding to the target molecule is eluted. Then, the eluted phage is used to infect new *E. coli* cells to amplify selected clones. This new phage library can be used in a new round of selection. Variants of this classical phage display are the so-called phagemid display[124] or bacterial surface display.[126–128] Examples of in vitro cell-free display techniques are the ribosome display[113,129] and mRNA display.[129]

Which selection technology is best suited for a given binding protein library depends on different parameters: the library diversity, the properties of the scaffold, and the intended applications.[113] Once selected, the combinatorial proteins are purified by a variety of methods.[124]

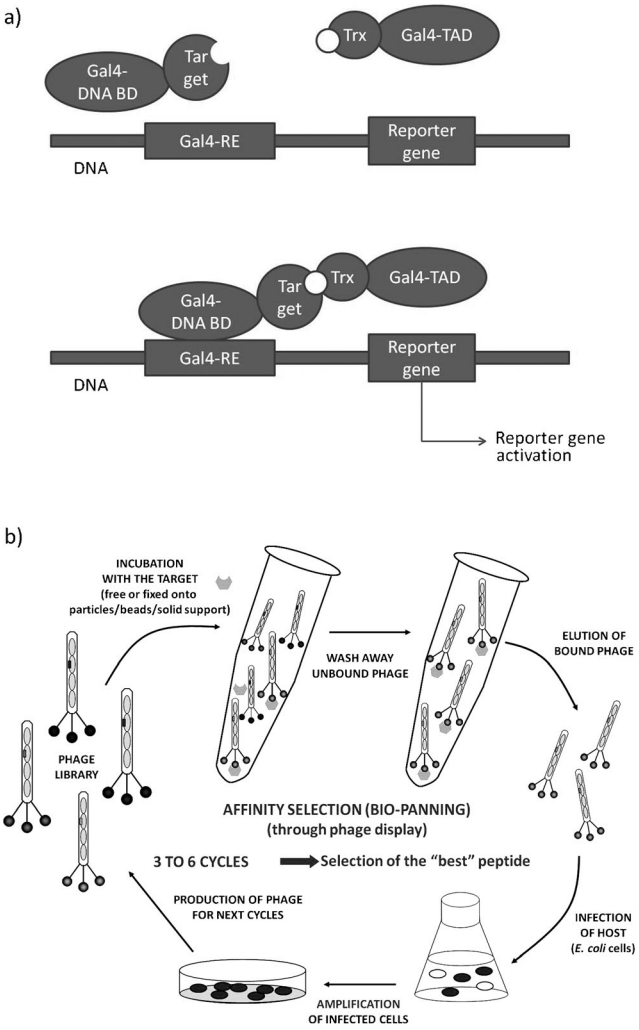

Figure 5. Selection systems: a) In the yeast two-hybrid system, the Gal4 transcription factor of *Saccharomices cerevisiae* is separated into a DNA binding domain (BD) and transcription activating domain (TAD). The target protein is fused to the DNA BD, the peptide inserted into a scaffold and fused to the TDA. Following interaction with the target peptide, the transcription factor will bind to *GAL4* RE and activate the transcription of different reporter genes. A second variation based on bacterial LexA protein can also be used (not shown). b) The phage display biopanning method is an in vitro system, in which the recombinantly expressed target protein is coated on a solid support and incubated with phages displaying randomized peptides on surface proteins. Target-binding phages are amplified and screened in successive rounds of positive selections.

4. Application of Nucleic Acid Aptamers

The enormous potential of aptamers as therapeutics has been extensively explored, and culminated in 2004 with the approval by the Food and Drug Administration of Eyetech/Pfizer's aptamer, Macugen, for the treatment of exudative age-related macular degeneration and diabetic macular edema.[130–134]

Without doubt, the potential use of aptamers as therapeutics, for drug delivery, and for in vivo diagnostics were the driving force for research on these molecules.[135–152] However, another important field of application of the aptamers is as bioreceptors in bioassays, as demonstrated by the high and increasing number of publications on this subject.[153–161] Aptamer-based bioassays can be set up in a wide variety of formats (direct, sandwich, or competitive). The main differences between the different formats are the immobilized species (aptamer, antibody, or target analyte), the number of experimental steps involved, and in which order the different reagents are exposed to the solid support, when present. The choice of the format depends on the molecular size of the analyte, the availability of reagents, and the cost. When it is possible to perform different assay formats for the detection of the same target analyte, it is useful to compare the analytical performances of each, to choose the approach that is the best compromise in terms of sensitivity, specificity, analysis time, and cost.[162,163]

Despite the large number of selected aptamers for many different molecules, published studies on aptamer-based assays show, however, that only a few specific aptamers have been used, therefore limiting the application of the assays and demonstrating that the proposed approaches often can not be generalized to all the available aptamers but are strictly related to the aptamer sequence and structure. Actually among the hundreds (>900) of publications on aptamer-based assays, sensors, or biosensors in the last ten years, almost 60% are dominated by only eight aptamers (see Figure 6). The thrombin aptamer represents the majority of this number: this point has been well considered by Baird,[164] who has defined it as "the thrombin problem". In the same publication Baird says that "aptamers have become, in some sense, the victims of their own success". Actually, the fact that only a few of the selected aptamers are currently used in the development of bioassays, demonstrates that the manipulation of well-known aptamers is much easier and more fruitful than bringing other aptamers that target more clinically relevant proteins to a full validation for analytical application. The impression is that researchers have carried out great work in selecting a plethora of aptamers and making the selection process faster, easier, and more widely applicable: it now seems that it is up to researchers focused on assay development to demonstrate that aptamers can replace, or at least join, antibodies in clinical applications. Our feeling is that one of the problems is the lack of easy and universal rules for the application of aptamers in bioassays, rules that have nowadays been well established when working with antibodies. The major difficulty when developing aptamer-based assays is, in this sense, the need for a systematic study on aptamer binding conditions, structure, and behavior. Moreover, the transfer of optimized parameters from one aptamer to another is sometimes impossible.

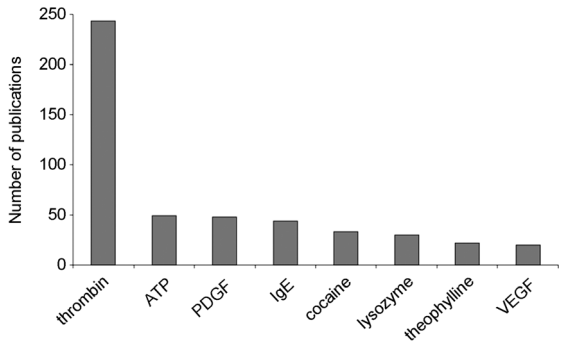

Figure 6. The most frequently used aptamers for biosensing.

Several examples of aptamer-based assays are highlighted in the following sections: the aim is to give an overview of the assays developed by using aptamers, concentrating mainly on studies based on aptamers other than the thrombin aptamer. The publications are reviewed by considering the assay format (direct, sandwich, and competitive assays), exploitation of the different approaches, in some cases with a critical and systematic study on the aptamer, and on the working conditions.

4.2.1. Aptamers in Single-Site (Direct) Assays

The high sensitivities required by the aptamer-based assays for the detection of some of the target analytes (e.g. pM level), often cannot be reached by a direct or single-site format, since the affinities of aptamers for their targets is not high enough (ranging from the micro- to the nanomolar level). For this reason, several strategies have been used as signal amplification tools, such as metallic and magnetic nanoparticles, enzymatic labels, and quantum dots.[153,165]

Aptamer-functionalized metallic and magnetic nanoparticles have been widely used for the direct detection of proteins and other molecules.[166–170] Various strategies have been developed for colorimetric assays based on aptamers and gold nanoparticles for direct format type assays. The major advantage of colorimetric assays based on aptamer–gold nanoparticles (AuNPs) is that molecular recognition events can be transformed into color changes, which could be monitored by absorption spectroscopy or visual observation; thus no sophisticated instruments are required. Based on this strategy, a method for target detection via an aptamer hybridized with a short complementary oligonucleotide attached to AuNPs at a specific salt concentration was presented.[171] The surface charge density of AuNPs could also be controlled through changes in the aptamer conformation by folding and unfolding on the AuNP surfaces. It was found that folded aptamer-modified AuNPs were more stable toward salt-induced aggregation than those tethered to unfolded aptamers. Based on this fact and on the predictable structure switching of aptamers, the analysis of adenosine was successfully realized.[172]

In a similar way to gold and magnetic nanoparticles, quantum dots (QDs) have been coupled to aptamers for the recognition of proteins.[169,173,174]

Other types of nanomaterials, such as carbon nanotubes (CNTs), used for signal generation and amplification have been used in combination with aptamers.[169,175,176] The photophysical properties of single-walled NTs (SWNTs), which act collectively as quenchers for dyes, have been used in the development of a sensing platform with the thrombin

aptamer.[177] The same principle has recently been used for the development of an assay for the detection of ATP by the noncovalent assembly of dye-labeled ATP aptamers and SWNTs.[178]

Moreover, it has been reported that aptamers work much more efficiently than antibodies in CNT field effect transistor (FET) sensors.[179] More recently, the same research group used carbon nanotube field-effect transistors (CNTFETs) in a label-free sensor for the detection of immunoglobulin E (IgE) in the range 250 pM–160 nM.[180] The authors concluded that the proposed method possesses a better detection limit than other methods based on aptamer–IgE interactions.

The fact that some aptamers fold or make a conformational change upon associating with their molecular targets is an interesting mechanism that can be exploited in the design of new aptamer-based direct assays.

Various assays, especially electrochemical sensors, based on this approach have been used for the detection of different targets such as theophylline,[181,182] lysozyme,[183] botulinum neurotoxin,[184] adenosine,[185,186] cocaine,[187,188] or thrombin.[189,190] In the electrochemical approach, the interaction of a labeled aptamer with its target can modulate the distance of the electroactive labels from the sensor electrode, thereby altering the redox current.

Despite the high number of published assays based on this approach, most of the selected aptamers are highly folded and fail to undergo any significant conformational change upon target binding, as recently discussed by Plaxco and coworkers.[191,192] Generally, when the conformational change is absent or partial and it does not generate any signaling event, a change in the aptamer geometry is necessary through the introduction of an antisense oligonucleotide which hybridizes with the aptamer, thereby keeping it in the unfolded form in the absence of the target,[193] or by destabilization of the native aptamer fold by truncation or the introduction of point mutations.[194] These aptamer engineering approaches have been explored systematically and compared by using two representative aptamers (ATP and IgE aptamer). It was observed that the relative change in the signal upon target binding varies by more than two orders of magnitude across the various investigated constructs and that the optimal geometry is specific to the aptamer sequence upon which the sensor is built.[192] An alternative possible alteration of the aptamer geometry has recently been proposed, which exploits the splitting of the aptamers into two suitable segments.[195]

The same principle—defined as the formation of supramolecular aptamer complexes (Figure 7)—has recently been used for the detection of cocaine by different research groups, who used electrochemical (voltammetry, impedance, and ion-sensitive field-effect transistor (ISFET)), photoelectrochemical, and SPR techniques.[196–199] The detection limits of the different configurations are in the range 1×10^{-6}–1×10^{-5} M[196–198] or lower (0.1 μM).[199]

4.2.2. Aptamers in Double-Site (Sandwich) Assays

The use of a sandwich format allows the target analyte to be detected with very high sensitivity and selectivity. Two conditions are required: 1) the analyte possesses two epitopes which are so different that both receptors can bind to the analyte without the binding of one affecting the binding of the other; 2) two aptamers are selected against such an analyte. The main disadvantage related to this format is that very few molecules (thrombin and PDGF) possess two aptamers that bind to two different sites and not all of the molecules have two binding sites. To overcome this fact, many authors have developed aptamer-based assays by using either the same aptamer as the primary and secondary ligands,[200,201] or an aptamer and an antibody as ligands for the sandwich.[202–205] The sandwich approach based on the use of two different aptamers has been reported mainly only for thrombin through the use of electrochemiluminescence,[206] magnetic beads and quantum dots,[207] or magnetic beads in an electrochemical assay.[208] The sandwich assay has also been performed on disposable microfluidic devices, fabricated on double-sided adhesive tapes and polymeric materials by using a laser cutting approach.[207] In this study a detection limit of 10 ng mL^{-1} was determined, with a linear range of 100–1000 ng mL^{-1} and an average standard deviation of 8%.

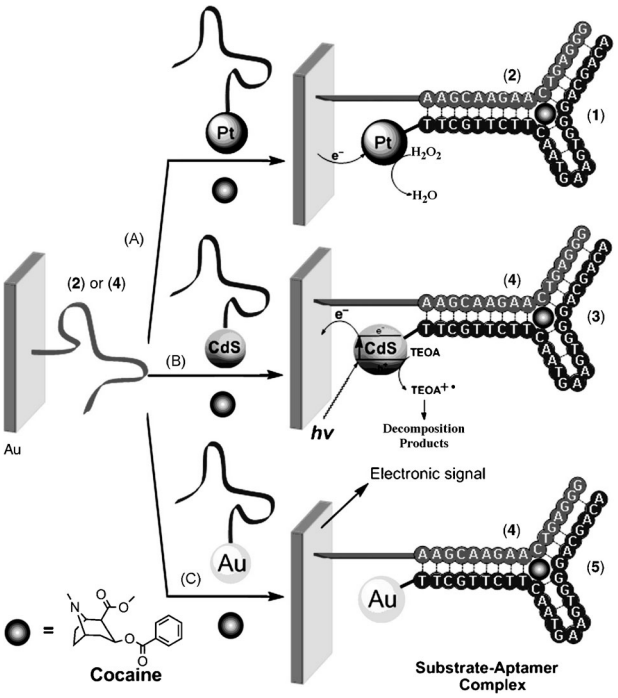

Figure 7. Electrochemical (A), photoelectrochemical (B), and SPR (C) detection of cocaine through the self-assembly of cocaine aptamer subunits functionalized with supramolecular complexes of platinum nanoparticles, CdS nanoparticles, or gold nanoparticles and gold surfaces functionalized with the second cocaine aptamer subunit in the presence of cocaine. Reproduced from Ref. [196].

4.2.3. Aptamers in Competitive Assays

The advantages of a competitive format are mainly related to the fact that only one aptamer is required (since two or more aptamers are not selected for many target analytes) and the time necessary for the assay is less. As an example, a disposable electrochemical competitive assay for

the detection of IgE has been reported.[209] In this study, the IgE antigen was immobilized on the surface of screen-printed electrodes, and then a competition step between IgE bound to the electrode surface and IgE in solution for the biotinylated aptamer was conducted. The detection limit was found to be $(23 \pm 4)\ ng\,mL^{-1}$ and the RSD 5.7 ± 0.8. In further studies, impedance spectroscopy (faradic impedance spectroscopy, FIS), was used as a transduction technique for a competitive aptamer-based assay for the detection of neomycin B.[210] The interesting feature of this study is the possibility of easily detecting a small molecule such as neomycin B with an electrochemical aptamer-based assay as an alternative to time-consuming label-based immunoassays or HPLC methods. A similar approach was followed for the development of an optical sensor for the same molecule, neomycin B, by using surface plasmon resonance (SPR).[211]

5. Application of Peptide Aptamers

Medical therapy and in vivo diagnostics are important fields of application for peptide aptamers.[4,96,105,212–219] Several artificial combinatorial proteins are in preclinical studies and a few of them are in clinical trials.[4] Theoretically, all the different artificial combinatorial proteins, provided that are able to bind a particular target molecule with sufficient affinity and selectivity, are applicable as bioreceptors in bioassays. To date, only a few studies have focused on the actual use in this regard.[101] In comparison to what happens in the nucleic acid aptamer field, the publications that deal with analytical applications are in the minority, with a prevalence for targeting medical diagnostic applications. To our knowledge, environmental applications of these affinity combinatorial proteins have been proposed as proof of concept, but not yet really tested, such as the possibility of using anticalins for the direct detection of low-molecular-weight compounds such as nonsymmetrically substituted phthalate esters.[101]

Different studies are reported in the literature in regard to diagnostic applications. These are reviewed on the basis of the scaffold used. Davis et al. used peptide aptamers based on the STM scaffold[220] to establish an SPR assay that offered a detection limit of 1 nM ($150\ ng\,mL^{-1}$) and determined the affinity constant of interaction of STM for a cognate antibody to be $K_D = (1.47 \pm 0.23)$ nM. The authors demonstrated that the STM scaffold mutated to introduce a single cysteine residue (STM^{cys+}; Figure 8a) enables direct immobilization on gold surfaces through formation of an S–Au bond (Figure 8b).

The same authors[221] presented an extension of this study, in which they used peptide aptamers to detect cyclin-dependent protein kinases (CDKs) and to optimize an immobilization procedure by using a homo-bifunctional maleimide cross-linker for conjugation between the cysteine residue and the sulfhydryl groups exposed on a thiol-functionalized surface.

Recently, the same research group[222] described another immobilization procedure for obtaining an oriented peptide aptamer surface and its utilization in establishing a highly specific, low-nanomolar-sensitive, SPR-based detection protocol for the active form of CDK2. In particular, they optimized a procedure based on a gold surface activated with [2-(2-pyridinyldithio)ethaneamine] (PDEA; Figure 8c). Significantly, the selected aptamers were able to detect subtle changes in the conformation of CDK2 associated with the activation of its catalytic activity. A typical response toward the inactive form of CDK2 was in the range of 0.5–2% of the binding of the active form of CDK2 in the concentration range from 2 to 20 nM, thus demonstrating that a non-antibody protein probe was able to detect an isoform of the active protein. This result raises the possibility that peptide aptamers will be able to extend the repertoire of probes that recognize protein conformations, post-translational modifications (PTMs), or conformations stabilized by PTMs.

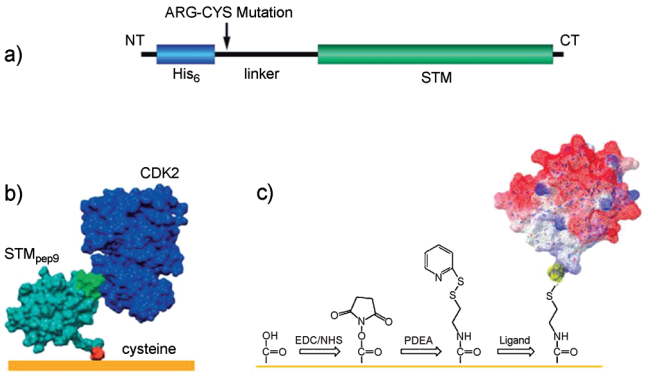

Figure 8. Examples of immobilization procedures using cysteine-modified STM-based aptamers (STM_{pep9}^{cys+}): a) schematic diagram of His_6-Cys-STM fusion protein illustrating the location of the introduced cysteine residue at the amino terminus of STM (reproduced from Ref. [221]); b) schematic diagram of the oriented STM_{pep9}^{cys+}-CDK2 complex[223] immobilized on the surface through an S–Au bond; c) surface activation by the PDEA protocol.[222] In this case, the gold surface was preactivated with a mixed self-assembled monolayer of (1-mercapto-11-undecyl)tri(ethylene glycol) and $HS(CH_2)_{10}(OCH_2CH_2)_3$-$OCH_2COOH$ (100:1).

The potential use of these scaffold proteins as capture probes in array formats has also been reported. The approach proposed by Walti and co-workers[223] is particularly interesting since they reported label-free detection techniques. The authors presented a procedure based on peptide aptamers, as artificial protein detectors arrayed on gold electrodes, and electrochemical impedance spectroscopy (EIS). They described a method to immobilize specific peptide aptamers on individual electrodes by using a masking/unmasking procedure based on methyl-terminated poly(ethylene glycol)$_6$-thiol and $STM^{(cys+)}$ on a gold electrode. EIS was used as a label-free, electrochemical method that monitors local variations in the impedance of the electrochemical layer above the surface of the gold electrode. The capture of protein molecules by an aptamer-functionalized electrode perturbs the electrical properties of the layer and thus its impedance. The authors demonstrated the specific recognition of CDKs in whole-cell lysates by using arrays of ten electrodes functionalized with individual peptide aptamers, with no measurable cross-talk between the electrodes. The sensitivity reported was within the clinically relevant range and can detect proteins against the high, whole-cell lysate background. Estrela et al.[224]

reported another label-free detection scheme for STM-based aptamer–protein interactions. By using accurate differential voltage instrumentation, they demonstrated a direct measurement of variations in the open-circuit potential (OCP).

Affibody molecules have also been investigated as affinity probes in protein microarray formats. For example, affibody molecules with affinity for IgA, IgE, IgG, TNFα, insulin, and Taq polymerase, were immobilized on thiol dextran microarray slides and then incubated with fluorescently labeled analyte. This revealed specific binding of the respective target protein with no observable cross-reactivity and a detection limit as low as 70 fM for the best affibody molecule.[96] Affibody molecules were also evaluated as capture agents in a sandwich array with unlabeled target protein and monoclonal antibodies used for detection, thus demonstrating specificity in a complex serum sample.[96]

Friedman et al. demonstrated the development of an affibody molecule that is able to simultaneously bind two different target analytes, namely HER2 (human epidermal-growth-factor receptor-2) and EGFR (epidermal-growth-factor receptor).[225] The simultaneous binding to two cell lines expressing the receptors was shown both in a microarray format and in real-time analysis of cell–cell interactions.

Affibody molecules have also been coupled to fluorescence resonance energy transfer (FRET) for the detection of analytes in solution. In Ref. [226], two different affibody molecules with affinity for either human IgA or IgG were produced by solid-phase peptide synthesis, thereby enabling site-specific conjugation of different fluorochromes at opposite ends of the affibody molecules. Adding target protein to the doubly labeled affibody molecules resulted in a concentration-dependent shift in the fluorescence ratio, induced by the binding of target protein and reduction in FRET between the acceptor and donor fluorophores. In a similar study by the same research group,[227] two different anti-idiotypic affibody pairs—consisting of an idiotypic antitarget affibody molecule and an anti-idiotypic affibody molecule competing for the same binding site as the target protein—were used for the detection of unlabeled target protein in solution.

Xu et al.[110] described the detection of TNF-α and leptin by using fibronectin III. Scaffold proteins were immobilized to predefined positions on a glass slide through specific base pairing between surface-attached oligodeoxynucleotides complementary to the DNA linker in the mRNA–protein fusions that were directly obtained from the selection approach. By using a sandwich-based detection format (using a Cy3-labeled antibiotin monoclonal antibody), the two investigated fibronectin variants were demonstrated to find their respective positions and selectively bind their targets.

In general terms, "peptide aptamers" could offer increased selectivity in detection applications for several reasons, as described before, but in principle for their specific binding.[110] Moreover, a small surface area of the scaffold reagent, in comparison with antibodies, should result in lower background signals arising from unspecific interaction with regions that are not directly involved in analyte recognition.[110] In assay formats involving the immobilization of a first affinity reagent for analyte capture, the use of such small reagents can in principle result in higher molar coating densities (i.e. binding sites per surface area) compared with large antibodies. Nevertheless, only a few studies, to our knowledge, discuss the behavior of these molecules in complex matrices such as biological fluids, and a detailed evaluation of the behavior of the main part of these molecules in real samples is still necessary.

6. Current Challenges and Trends

As discussed in the previous sections, aptamers (both nucleic acid as well as peptide) are gradually entering the arenas of classical antibody applications. This is also underlined by the commercial exploitation of some of these molecules for different applications. Medical therapeutic and diagnostic are still the major areas of interest and use. However, these new classes of reagents are also of interest to other biotechnological fields.

The possibility of using nucleic acid and peptide aptamers as bioreceptors in bioassays is demonstrated by the vast number of publications; however, a great number of these studies report analysis only under standard conditions; further information regarding their behavior in real matrices are still needed. Nevertheless, some recent studies have tackled this issue and show that aptamers can be successfully used in clinical specimens. From these we will cite the papers of Gold et al.[80] and Ostroff et al.,[228] where SOMAmers were used in multiplexed proteomic technology for biomarker discovery. In particular in Ref. [228], the authors described the identification of 44 biomarkers by comparing the sera of heavy smokers not known to have non-small-cell lung cancer (NSCLC) or known to have benign nodules with the sera of heavy smokers known to have either early stage or late stage NSCLC. The data for the entire experiment were collected from serum samples of 1326 patients (with 870 protein measurements per sample) from four independent biorepositories. More recently, in 2011, Muller et al.[229] reported an interesting assay for the detection of thrombin in plasma samples obtained from 20 healthy blood donors and controlling carefully the preanalytical conditions. Again, Tan and coworkers[63,230] have shown the applicability of aptamers as bioreceptors for both the extraction and the enrichment of tumor cells (TC) from body fluids, such as blood or sputum, as well as their detection.

Another crucial aspect that should be overcome for a full exploitation in bioassays is the application to environmental as well as food samples. In this respect, some examples have been reported for nucleic acid aptamers but only a few for "peptide aptamers". In regard to peptides, the use of scaffolds based on GFP or β-lactamase, which naturally exert a defined spectroscopic or biochemical activity, could be particularly interesting in bioassays since they are characterized by an integrated binding and reporting function, thus allowing direct quantification of the target by measurement of the fluorescence or enzyme activity. However, this possibility has not yet been fully exploited. A discussion of the challenges of aptamer evolution technology is beyond the scope of this Review; however, the development of LNA (locked nucleic

acid) aptamers[231,232] and PNA (peptide nucleic acid) aptamers[233] can benefit the field of biosensing of molecules through their maximum chemical diversity, minimum size, and high biostability. Similar considerations can also be envisaged for peptide aptamers by the inclusion of non-natural amino acids that will increase biostability and introduce new functionality and properties.[234]

7. Summary and Outlook

Nucleic acid aptamers and "peptide aptamers" have the common feature of a combinatorial nature. This fact greatly increases the possibility of finding new binding molecules. Their stability, their high yield of production and their synthetic nature that allows the use of animals to be circumvented for their production, are all very interesting properties that should help to increase their use in different fields of science.

Although the advantages of aptamers versus antibodies are evident, it is hard to compare nucleic acids and proteins. Proteins possess a variety of functional groups not present in nucleic acids which can enhance interactions and thus the affinity with the target (i.e. through the formation of hydrogen bonds or electrostatic bonds). Proteins have a different acid–base behavior than nucleic acids. The more rigid backbone of proteins compared to nucleic acids may also be an important advantage. As stated by Wilson and Szostak regarding a comparison between enzymes and ribozymes,[46] "*a protein active site may have around 8 amino-acid positions that directly contribute to substrate binding, and thus the number of possible combinations of side chains that evolution could sample would therefore approach 10^{10}; a ribozyme active site would be much more restricted in the number of different combinations of side chains and functional groups that it could utilize*". Clearly, this consideration can be translated to all classes of nucleic acid aptamers and proteins, including peptide aptamers. Nevertheless, a comparison of these two classes of affinity molecules is not a trivial matter. Different considerations have to be kept in mind to make this comparison, such as selection or production procedures, which can lead to different evaluations.

Moreover, from an analytical point of view and considering their application as reagents in bioassays, the nature of the sample (i.e. clinical specimen versus environmental matrices) must not be forgotten, as it can greatly influence the performance of a molecule with respect to another. Thus, it is impossible to predict which affinity molecule is the best for a particular application.

However, it is our opinion that the availability of a plethora of binding molecules, such as nucleic and peptide aptamers, which are different in chemical composition and produced by different ways, provides huge advantages in bioanalysis.

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