1 Is less more? Lessons from aptamer selection strategies

Zsuzsanna Szeitner, a Judit András, a Róbert E. Gyurcsányi, b Tamás Mészáros a,c

a Department of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University
Tűzoltó u. 37-47, H-1094 Budapest, Hungary
E-mail: meszaros.tamas@med.semmelweis-univ.hu

b MTA-BME “Lendület” Chemical Nanosensors Research Group, Department of Inorganic and Analytical Chemistry, Budapest University of Technology and Economics
Szent Gellért tér 4, H-1111 Budapest, Hungary
E-mail: robertgy@mail.bme.hu

c MTA-BME Technical Analytical Chemistry Research Group of the Hungarian Academy of Sciences
Szent Gellért tér 4, H-1111 Budapest, Hungary

1. Introduction. Antibodies versus aptamers

Biomarkers always have been in the focus of diagnostics and therapeutics, and their exploitation in clinical trials and medical practice is steadily increasing. Although previous research activities focused on nucleic acid biomarkers, which led to the development and wide application of platforms for high-throughput analysis of DNA variants and mRNA expression profiles, it has been recognized that analysis of protein biomarkers provides larger amount of relevant information. Progress of proteomics technologies has brought about the explosion of our knowledge in the field of disease-related protein patterns, and thousands of proteins have been documented as biomarker candidates [1]. Thus, importance of selective detection and targeting of individual proteins can hardly be overestimated. Presently, the antibody-based assays are the most sensitive, specific and selective methodologies for detection and
characterization of proteins. Consequently, public domain initiatives have been launched to
deposit antibodies against all human proteins in databases with free accessibility (e.g. HUPO
Antibody Initiative) [2].

Pivotal role of antibodies is not restricted to selective recognition of proteins since their
application is also inevitable in routine diagnostics of small molecules such as antibiotics,
hormones, and food toxins [3]. To meet the receptor demand of therapeutics and diagnostics,
a vast number of antibodies have been produced and various improvements have been made to
their generation. However, application of antibodies is inherently limited by their susceptibility
to environmental conditions, immunogenicity, and in vivo production. Therefore, there is a
continuous quest for appropriate alternatives of antibodies.

It has been long known that single stranded RNAs (ssRNA) form elaborate 3D structures
in ribosomes. Recent discovery of riboswitches has also revealed that several mRNA molecules
could selectively recognize and bind to their matching metabolites, functioning as ancient
bioprobes, predecessors of protein receptors [4]. In a similar manner, the short, single stranded,
in vitro selected DNA or RNA molecules, the so called aptamers also assume specific secondary
structures and oriented conformations, which allows them to selectively bind their target
molecules (Figure 1). The significance of aptamers resides in the possibility of directed
generation of these oligonucleotides for selective binding of theoretically any targeted
compound. The methodology of in vitro selection of oligonucleotides was published almost
simultaneously by two independent research groups in 1990. The term aptamer has been coined
in an article by Ellington and Szostak in Nature [5], while that of “SELEX” (Systematic
Evolution of Ligands by EXponential enrichment) first appeared in a paper in Science authored
by Tuerk and Gold [6].

Figure 1.
While the best dissociation constants of published aptamer–target complexes seem to be similar to those of antibody–antigens, aptamers are superior to antibodies in several aspects [9]. These advantages of aptamers can be attributed to their chemical properties and *in vitro* selection, and chemical synthesis. Oligonucleotides are conveniently prepared with high reproducibility and purity; therefore, no batch-to-batch variation is expected in aptamer production. Moreover, they withstand long-term storage at ambient temperature while preserving their functionality, which can be tailored on demand during chemical synthesis, e.g., to aid their immobilization, to impart signaling properties, and/or to increase their resistance to enzymatic degradation. Finally, the low immunogenicity and small size of aptamers are appealing advantages with respect of their therapeutic and diagnostic application. Although all these properties contribute to the growing popularity of aptamers, their *in vitro* selection could probably be highlighted as their most important strength.

The obvious consequence of the living organism-free selection method of aptamers is that it can be applied where antibody raising would fail, i.e., aptamers can be selected for toxins as well as for molecules that do not elicit adequate immune response, which outlines the universal character of the aptamer selection concept [10]. Antibodies are generated in cells and prone to lose their activity under non-physiological conditions that restricts their diagnostic utility. On the contrary, application of aptamers is not limited to physiological circumstances since their selection conditions can be adjusted so as to be equivalent with those of the proposed *in vitro* diagnostic exploitation. Additionally, the kinetic parameters such as the on- and off-rates of aptamers could also be finely tuned according to the requirements of the detection method. A further merit of aptamers is their extreme selectivity that enables them to discriminate molecules with slight structural differences or even the enantiomers of chiral target molecules, such as amino acids and drugs [11-13].
2. Aptamer selection

2.1 Basic principles

Like most of the groundbreaking ideas, the theory of SELEX is very simple, relying on Darwinian evolution at a molecular level. Basically, a vast number \((10^{14}-10^{16})\) of DNA or RNA oligonucleotides with different sequences are subjected to selection for binding to the target molecules. The classical SELEX methodology involves the immobilization of the target compound on a solid support, which is then brought in contact with the pool of oligonucleotides. While non-binding oligonucleotides are discarded by washing steps, the bound sequences exhibiting affinity for the target are amplified by PCR. The multiplied, double stranded DNA is either converted into ssDNA or used as template for \textit{in vitro} transcription and the enriched oligonucleotide library is reintroduced in the follow-up selection cycle. Generally, after 8–15 cycles, the oligonucleotide pool is populated by the best binding aptamer candidates, which are finally separated and identified by sequencing. The first cycle is decisive for the success of the whole selection process because hypothetically the oligonucleotide of any possible sequence is represented only as a single copy in the starting degenerate library. Accordingly, for the initial round(s) of selection, longer incubation times and less stringent conditions are applied and these parameters are gradually changed during the subsequent cycles to increase the “selection pressure”.

The first studies on aptamers involved mostly RNAs, motivated mainly by the assumption that RNA can form more diverse 3D structures than DNA, which is believed to be beneficial in terms of establishing a higher affinity to the target. However, the RNA SELEX is more complex than the DNA SELEX [14] owing to the fact that additional \textit{in vitro} transcription steps are needed before and after each PCR amplification. Additionally, the RNA molecules
are prone to enzymatic degradation, which is a major problem to be addressed in most applications. The authors of this review are not aware of any systematic study indicating a higher affinity of either type of aptamers. The fact that both RNA and DNA aptamers are frequently reported to form complexes of submicromolar or even subnanomolar dissociation constants with their ligands further challenges the assumption of a marked difference between their affinities. Beside the natural nucleic acids, RNA and DNA libraries containing various modified nucleotides were also used for generating aptamers. Although the primary motivation of these efforts was to increase the nuclease resistance of oligonucleotides, several modifications also conferred aptamers with higher affinity [15]. Recent innovations have added functional groups that mimic amino acid side-chains to expand the chemical diversity of aptamers [16, 17]. These latest developments have eliminated one of the drawbacks of conventional aptamers, namely the lack of hydrophobic moieties. This resulted in drastically increased success rate of selection and yielded aptamers with subpicomolar affinity. Of note, both publications have reported application of modified deoxynucleotides prognosticating the dominance of DNA aptamers in the future.

Figure 2.

Implementation of aptamer production is much more complex than its simple, theoretical scheme (Figure 2) would suggest, and the success of the procedure mainly relies on seemingly minor experimental details of the selection. Consequently, following the introduction of SELEX, numerous alternative approaches have been explored [18] with the general intention of increasing the success rate, but also ensuring high speed [19, 20], low handled volumes [21], minimal contamination and automation [22].
The conventional SELEX procedure needs high purity targets to ensure the selectivity of isolated aptamers. In the case of proteins, this condition is generally fulfilled by using recombinant proteins with various fusion tags (e.g. polyhistidine and glutathione S-transferase (GST)). The fusion tags do not only simplify the purification protocol from the protein overexpressing cell culture or *in vitro* translation system, but they also enable oriented immobilization of the targets during the SELEX process; thus, the desired epitope of the protein could be readily exposed for aptamer generation.

Even if absolute purity of the target protein is assumed, the selection is complicated by the contingent binding of oligonucleotides to the solid support and the cross-linker used for immobilization. Therefore, the so called counter selection by which sequences that show cross-reactivity to the matrix components are discarded is of utmost importance in the selection of highly selective aptamers. The counter selection is a major asset also in developing aptamers for well specified analytical or therapeutical tasks by eliminating cross reactive aptamers to all known critical interferents of the sample. Thus, with proper background information on the support and sample matrix to be involved a more rational selection is possible. Various development have been made that enable production of aptamers with the desired high selectivity; however, the opportunities offered by these striking advantages of aptamer-based assays seems to be less appreciated, as the analytical reports in general do not employ custom-selected original aptamer sequences.

2.2 Increasing the selectivity

One of the first classical aptamer publications has already demonstrated that the basic selection method could provide aptamers, which could discriminate among organic dyes with very similar chemical structures [4]. Since then, panel of modifications have been made to the
original protocol to increase further the selectivity of generated aptamers. The first improvement has been described in the publication that presented the selection of DNA aptamers for the first time [11]. The authors followed their previous protocol used for the isolation of organic dye selective RNA aptamers, but when the pools that had been selected for three cycles were applied to non-cognate dye columns, the ssDNA pools bound to every tested dye, i.e., no selectivity was observed. Apparently, the oligonucleotides were nonspecifically retained, either because of binding to the agarose matrix or universal dye binding. To remove nonspecifically binding sequences, negative selection has been introduced, that is the selected ssDNA pools of third cycle were flown over the non-cognate dye modified columns prior to next positive selection cycle, which resulted in the removal of the sequences showing cross-selectivity from the selection library. This simple negative selection cycle significantly increased enrichment of selectively binding oligomers, and has been routinely applied during aptamer selection since its introduction.

Soundness of this rationale was further validated by production of an RNA aptamer that binds theophylline with 10,000-fold greater affinity than caffeine, which differs from the target molecule only by an extra methyl group [8]. The aptamers were isolated by addition of the RNA pool to theophylline coupled Sepharose column and the stringency of selection was increased by removing of non-specific binders by washing the column with caffeine before collection of theophylline selective oligonucleotides. This modified version of negative selection was designated counter SELEX. Another outstanding example of discriminating capacity of aptamers was also demonstrated by using negative selection combined with harsh washing conditions to isolate arginine specific oligonucleotides [9]. The protocol involved a counter selection with citrulline, but to increase the stringency of competition between free citrulline and immobilized arginine, the column bound RNA was heat denatured and renatured in the presence of citrulline before elution with arginine. This rigorous selection scheme led to a tight
binding RNA aptamer, which discriminates 12,000-fold between the D- and L-enantiomers of
arginine. It should be noted that confusingly, the negative and counter selections have been
widely used as synonymous expressions in the aptamer related publications.

The success of negative and counter selection hinted that beside highly purified proteins,
complex heterogeneous targets are also suitable for generation of specific aptamers. An
important practical application of this theoretical possibility, the so called Cell-SELEX method
isolates cell type specific aptamers by following the above described rationale. It combines
positive and negative selection steps during the selection procedure but uses whole cells instead
of immobilized molecules as targets of aptamers. The most remarkable advantage of this
approach is that cell-specific aptamers can be obtained without any knowledge as to the cell
surface molecules of the target cell. Due to the attractive features of this approach, many
variations of Cell-SELEX have been developed and a wide array of cells has been used as
targets of selection [23].

The SELEX most often involves utilization of recombinant proteins, and this could lead
to limited applicability of produced aptamers. Majority of eukaryotic proteins are post-
translationally modified and many of them are membrane integrated thus the proteins in their
native conditions are often differently structured from the recombinant variants. Due to the
discriminating capacity of aptamers, using the standard, one ligand SELEX, even a slight
difference of native and recombinant proteins may preclude identification of aptamers, which
maintain their functionality with their physiological targets. This shortcoming of SELEX has
been illustrated with isolation of E-selectin specific thioaptamers [24]. Amongst the 14
aptamers selected by using recombinant protein only one bound to endothelial cells expressing
E-selectin, even though the applied, human recombinant protein had been obtained from
mammalian system. This observation highlights that integration of biologically relevant
conditions into the screening process increases the success rate of identification of aptamers
with pertinent biological activity. In the last decade, the Cell-SELEX has become a routinely applied method; therefore, alternation of recombinant proteins and target protein expressing cells during the steps of selection procedure can be expected to become a more widely applied aptamer producing approach.

Considering the procedure of translation of lead molecules into therapeutic agents, the achievable, extremely high-selectivity of aptamers could be also a disadvantage, since the aptamers isolated for human proteins might possess low affinity for the homologous proteins of animal models and thus reduced in vivo efficacy. To ensure both the required selectivity and species cross-reactivity of aptamers intended for therapeutic applications, the toggle SELEX method was put forward [25]. Using this protocol, nuclease resistant RNA ligands that bind both human and porcine thrombin with similar affinity have been produced by changing, “toggling” the human and porcine protein during alternating rounds of selection. The selected aptamer also has been shown to increase thrombin time in both human and porcine serum clotting assays.

2.3 Selection without target immobilization

Improvements of the solid supports to minimize oligonucleotide absorption represent an important aspect in the development of SELEX variants. In any case, additional stringent counter-selection steps are needed to screen out those oligonucleotides that bind to the support. Immobilization of the target is also critical in terms of having exposed the desired epitope for aptamer generation. Therefore, from the plethora of alternative selection methodologies, the homogeneous approaches need to be highlighted owing to their advantage of not requiring target immobilization and, consequently, a solid support. These techniques are dominated by
electrophoretic methods, most notably by capillary electrophoresis [26] and free-flow electrophoresis [27].

Motivated by the higher efficiency partitioning of kinetic capillary electrophoresis (KCE) over traditional separation methods by at least two orders [28], capillary electrophoresis-SELEX (CE-SELEX) have been introduced to produce protein selective aptamers [23]. In CE-SELEX the aptamer-target interaction is performed in solution and the high resolving power of CE is used to separate unbound and target-bound oligonucleotides, the latter being collected and subjected to PCR amplification before being reinjected. Due to the high separation efficiency and rate of enrichment, high affinity aptamers are obtained in only 2–4 rounds of selection [29]. It has been documented that the selection could be distorted by intrinsic differences in the amplification efficiency of nucleic acid templates; hence, the most abundant oligonucleotides of SELEX do not necessarily represent the highest affinity aptamers [30]. Consequently, the reduced number of selection cycles of CE-SELEX not only shortens the time of aptamer production but also lessens the deleterious effect of extended number of PCRs of conventional SELEX. In order to further accelerate the selection procedure and to exclude the DNA amplification bias, repetitive steps of PCR have been completely omitted from the iterative cycles of selection [31]. This, so called non-SELEX protocol involves less than four repetitive steps of partitioning by KCE without any amplification between them and provides protein selective aptamers in less than a week.

To alleviate the PCR bias issue of aptamer selection procedure, a target immobilizing approach without iterative amplification cycles also has been developed [32]. MonoLex method relies on application of affinity capillary column coated with the selection target and physical segmentation of the column into slices following the chromatography of oligonucleotide library. The different column fragment bound aptamer candidates are separately amplified with a single PCR and their binding specificity is assessed by dot blot assay.
Although CE-SELEX and non-SELEX have been proved to be fast and effective ways of isolation of protein selective aptamers, application of these methods also have their own limitations. Since negative selection is not involved in KCE-based aptamer production, great purity of target protein is a basic requirement of successful identification of aptamers that are selective for the protein of interest. Thus, protein sample has to be thoroughly analyzed prior to its application. The CE-SELEX and non-SELEX protocols can be accomplished in a week; however, the optimal conditions of partitioning have to be determined individually for each protein, which could be a challenging task. Furthermore, thermal band broadening of CE due to Joule heating restricts the applicable ion concentration of partitioning buffers [33]; hence, the selection conditions might not be adjustable to the circumstances of prospective usage of aptamers [34]. Finally, one of the benefits of CE-SELEX, i.e., the small analyte requirement is accompanied with an inherent shortcoming of the approach. The typical sample injection volume in the range of nanolitres limits the sequence space that can be screened for target binding. This is contrary to the optimal selection conditions whereas oligonucleotides are added in large excess over the target molecule so that the probability of the presence of high-affinity aptamers is increased, and competition for target proteins facilitates isolation of the best binders from the pool.

Some of the above mentioned disadvantages of CE-SELEX such as sample volume limitation and selection buffer restrictions may be overcome by using the free-flow electrophoresis (FFE) technique in which the electrophoretic separation is performed on a continuous flow of analyte in a planar flow channel. In contrast to CE where the electric field is applied in the direction of the fluid movement, in FFE, the electric field is applied perpendicularly to the pressure-driven flow to deflect the analytes laterally according to their mobility [35]. Aptamers with low nM dissociation constants for protein targets were detected following a single round of selection with micro FFE [24]. The electrophoresis techniques have
driven an obvious progress in terms of reducing the selection time; however, apparently there
is no significant improvement in lowering the dissociation constants of the selected aptamers
as compared with conventional SELEX techniques. For instance, dissociation constants of the
aptamers selected for IgE using the conventional SELEX method were as low as 10 nM [36],
somewhat lower than those of aptamers obtained by CE-SELEX (~ 40 nM [23]) and by micro
FFE (~ 20 nM) [24].

2.4 Miniaturization of selection

In most of the traditional SELEX procedures, non-selective oligonucleotides are
removed from target molecules either via membrane filtration or column chromatography, or
binding of the target protein to the wells of microtiter plates [15]. Due to the low partitioning
efficiency of these separation methods and the binding of oligonucleotides onto the matrix of
stationary phases, isolation of high-affinity, selective aptamers requires typically 8-15
cumbersome selection cycles. A significant improvement has been made to the conventional
selection technology with introduction of paramagnetic beads for target protein immobilization
[22]. Paramagnetic beads offer advantages over column chromatography in their ease of use
even in the microliter range. Hence, very small amounts of target protein coated beads can be
rapidly partitioned, stringently washed, and the protein bound oligonucleotides can be
subsequently eluted. These benefits of paramagnetic beads have made the manual aptamer
selection faster, more straightforward, and provided DNA and RNA aptamers with high affinity
[37, 38]. Significantly, an automated aptamer selection process has also been established by
using paramagnetic beads [22]. The enhanced, fully integrated robotic system accommodates
all steps of the aptamer production including isolation and amplification of selective RNAs.
The reported workstation can carry out eight selections simultaneously and can complete 12
rounds of selection in two days [39]. The same research group improved the protocol even further by completing the system with *in vitro* transcription and translation of target proteins [40]. *In vitro* translation is an effective way of high-throughput production of proteins thus could serve as a supply of target proteins for aptamer selection [41]. Although these results could make one envision a fully automated pipeline of aptamer production from coding gene to protein-selective aptamer, the practical, high-throughput application of the combined system has not been published, yet.

A mathematical model describing the optimal conditions for SELEX has pointed out that strong competitive binding of oligonucleotides can yield the highest affinity aptamers [42]. To achieve the theoretically ideal ssDNA ratio, single microbead SELEX has been developed and applied successfully for isolation of botulin neurotoxin selective aptamers. However, manipulation of microscopic amount of beads demands delicate handling, thus it is not suitable for routine application [43]. The advanced microfluidics provide miniaturized sorting technologies for manipulation of individual particles or cells with continuous operation [44]. Realizing the benefit of these systems, a chip-based magnetic bead-assisted SELEX with microfluidics technology, so called magnetic SELEX (M-SELEX) has been invented [45]. Partitioning efficiency (PE) is a generally accepted indicator of the success of separation. Lou et al. have demonstrated that the PE of their continuous-flow magnetic activated chip-based separation (CMACS) device is ca. $10^6$, thus it significantly exceeds the efficiency of conventional separation methods, and is comparable to that of CE. They combined the outstanding PE of CMACS device with usage of carboxylic acid activated paramagnetic beads for target protein immobilization to reduce the nonspecific binding of negatively charged oligonucleotides onto the beads. The effective separation and low background binding of oligonucleotide library enabled isolation of Botulinum neurotoxin specific aptamer with low-nanomolar dissociation constant after a single round of selection. However, the use of the
CMACS needed scrupulous tuning of the device with microscopy to achieve the high PE and recovery of bead-bound oligonucleotides. To address this shortcoming, the research group converted the CMACS device into micromagnetic separation (MMS) chip, which is more robust and does not require a microscope for practical application [46]. Using the MMS chip, they optimized their previous CMACS-based protocol by determining the ideal buffer flowing rate, elevating the temperature of selection, and introducing a counter selection step.

Besides the excellent PE, a further benefit of MMS chip is its capacity to concentrate a small number of beads suspended in a large volume into a miniature chamber. This feature facilitates the implementation of the so-called sample volume dilution challenge technique wherein the target-aptamer complexes are equilibrated in increasing volume of buffer during the consecutive selection cycles to favor enrichment of aptamers with slow off rate. Soh et al., exploiting the concentrating capability of the MMS chip, have developed an aptamer selection protocol that combines the volume dilution challenge with high-stringency, continuous washing inside the chamber of the device. These improvements translated to isolation of aptamers with less selection cycles. Previously, streptavidin selective aptamers were generated by conventional magnetic bead-based SELEX with 13 selection cycles, while the MMS chip-based, enhanced protocol provided aptamers for the same target protein with even lower equilibrium dissociation constants ($K_D$) through 3 iterative steps [47].

Emerging of M-SELEX approach initiated mathematical remodeling of aptamer selection procedure and the obtained numerical data highlighted a further advantage of MMS chip-based method [48]. The authors compared the conventional filter-based SELEX and M-SELEX and their calculations have drawn the attention again to the importance of the non-specific, background binding of oligonucleotides onto the matrix of the stationary phase of the process. According to the proposed model, the fraction of high affinity aptamers reaches 100% at the 8th selection cycle with the low background binding M-SELEX, while the application
of filter for separation yields merely 12% of high quality aptamers at the same round of selection. The reduced number of iterative steps apparently implies faster aptamer producing procedure, but more importantly, it also drastically decreases the enrichment of non-target selective oligonucleotides resulting from intrinsic differences in the amplification efficiency of nucleic acid templates.

Although it has been both theoretically and experimentally demonstrated that keeping the background binding at minimum is a prerequisite of the productive aptamer selection, density-dependent cooperative (DDC) binding also has to be taken into consideration to evade the isolation of aptamers with low affinity. DDC binding occurs when the ligand tethers concurrently to more adjacently immobilized targets in a cooperative mode that could increase the binding affinity by two orders of magnitude [49]. This phenomenon could deteriorate the aptamer selection by populating the enriched oligonucleotide library with concurrently binding aptamers. Considering the comparatively modest number of beads used in M-SELEX, DDC binding is a particularly important issue with the microfluidic aptamer selection devices. Therefore, the ratio of magnetic beads and immobilized protein has to be determined according to the compromise between background and DDC binding.

Table 1 summarizes the characteristics of the best aptamers obtained by the discussed methods. Closer examination of the data reveals that high-affinity aptamers can be selected with the traditional SELEX approaches as well, but these procedures demand more selection cycle thus cannot meet the requirement of an ideal, high-throughput receptor generating system.

### Table 1

| Characterization of aptamer candidates |  |

15
Although the success of aptamer production is mainly dictated by the careful planning, meticulous implementation and following of progression of selection [50], there is another remarkable aspect of SELEX receiving little attention from the end users of aptamers. Since most papers feature only a single aptamer, there is little awareness that the selection process generally results in a large number of sequences. Ideally, all selected oligonucleotides need to be evaluated individually in terms of their target binding properties to designate the most auspicious aptamer candidates. Actually, this characterization is one of the most costly and time-consuming tasks of the aptamer production. The sheer number of methods that have been used to determine the dissociation constant of aptamer-target molecule complexes speaks both the importance and difficulty of these measurements. The developed methods range from the low-cost, simple approaches such as dialysis and filter binding assays to surface plasmon resonance (SPR) and amplified luminescent proximity homogenous assay (ALPHA) requiring dedicated instrumentation[27, 51]. As Figure 3 shows, the applied methodologies have different sensitivities and requirements in terms of estimated analysis time and sample volume. The measurements are further complicated since post-selection labeling or immobilization of aptamers may significantly affect their binding distorting the $K_D$ of native aptamer. Additionally, the $K_D$ values obtained from different methods could be noticeably divergent [52] [53]. Considering all of these factors, $K_D$ values should be determined with applying a method that most closely simulates the circumstances in which the aptamer is intended to be used. Noteworthy messages of these hindrances are that affinity of aptamers is suggested to be measured by two different approaches and even the most carefully determined $K_D$ values have to be handled cautiously. Altogether, the practical value of aptamers cannot be revealed without their thorough evaluation in the proposed application.
4. Outlook

Aptamers have been around for almost a quarter-century; however, their versatile applicability was acknowledged only a decade ago. Since then, the aptamer related publications and the number of selective aptamers has been exponentially increasing, and the aptamers have appeared on the market, too. Although the theory of aptamer production has not been changed since its first description, various, crucial modifications have been made to the original SELEX procedure to enhance the effectiveness of selection. Due to these improvements, the recent aptamer producing methods require less time and protein, while allow high-throughput isolation of selective aptamers with high affinity [20, 54].

It is important to notice that, despite the evident bioanalytical potential of aptamers, their analytical applications started to appear with a considerable lag. The reason seems to be related to the lack of an experimental biological background required for aptamer selection in analytical laboratories. Therefore, the overwhelming majority of the analytically aimed studies were performed on a relatively limited number of well-characterized model aptamers, such as human thrombin in ideal samples. The biosensor development was long dominated by glucose biosensors taking advantage of the highly stable and cheap glucose oxidase enzyme to test and demonstrate different detection methodologies and materials. Thrombin has become the dominant target (analyte) in aptamer-based sensing essentially for similar reasons. More than 900 papers have been published on thrombin aptamers to date, which, given the versatility and almost universal use of aptamers for any target, is hard to be justified by the importance of thrombin–aptamer recognition alone. Although a limited number of aptamers have been used
for analytical studies, we have witnessed a tremendous development in the aptamer-based
analytical methodologies in the last decade. Most of the routine immunoanalytical
methodologies were seamlessly adapted to detect aptamer–ligand interactions [55]. Thus,
utilization of aptamers in label-free techniques such as SPR [56], SPR imaging [57], quartz
crystal microbalance [58, 59], microelectromechanical sensors [60], nano field effect transistors
(nanoFETs) [61], and electrochemical impedance spectroscopy [62], as well as in various
amplification schemes based on enzymes [63], luminescence-generating labels, and
nanoparticles [64, 65] have been demonstrated. Moreover, the range of bioassay methodologies
was further extended by exploiting the inherent properties of nucleic acid aptamers in molecular
beacons [66-68], ligation assays [69], electrophoresis [70], microarrays [71], and direct
reporting through the use of catalytic oligonucleotides (ribozymes and deoxyribozymes) [72].

Considering that the aptamer production pipeline has become an ideal system for
fulfillment the persistent demand of biomarker selective receptors, and their widespread
analytical application has also been demonstrated, aptamers are expected to be used for
detection of an expanding number of biomarkers and gain ground in routine diagnostics.

Acknowledgement

The financial support of ENIAC (CAJAL4EU), the Momentum (Lendület) program of the
Hungarian Academy of Sciences (LP2013-63/2013), and New Széchenyi Plan (TÁMOP-
4.2.1./B-09/1/KMR-2010-0001 and TÁMOP-4.2.1/B-09/1/ KMR-2010-0002) is gratefully
acknowledged.

References


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<td>300 nM</td>
<td>non-equilibrium capillary electrophoresis of equilibrium mixtures</td>
<td>31</td>
</tr>
<tr>
<td>Microfluidic selection</td>
<td>Botulinum neurotoxin type A, Transcription factor IIB, C-reactive protein</td>
<td>1, 7, 5</td>
<td>33 ± 8 nM, 4 nM, 3.51 nM</td>
<td>fluorescence binding assay, electrophoretic mobility shift assay, SPR</td>
<td>45, 19, 20</td>
</tr>
<tr>
<td>M-SELEX</td>
<td>Streptavidin, Platelet-Derived Growth Factor-BB</td>
<td>3, 3</td>
<td>25-65 nM, 0.028 nM</td>
<td>fluorescence binding assay, fluorescence binding assay</td>
<td>46, 47</td>
</tr>
</tbody>
</table>
Figure Captions

**Figure 1.** Elaborate 3D structures of aptamers. Crystal and solution structure of von Willebrand factor [7] and the malachite green [8] binding aptamers A and B, respectively.

**Figure 2.** Principle of the SELEX method to generate aptamers

**Figure 3.** Comparative bubble graph of various techniques used for determining the dissociation constant of aptamer–protein complexes. The graph shows the smallest assessed $K_D$ values for aptamer–protein interaction with the respective techniques as a function of the minimal volume required for the analysis.
Figure 3

![Diagram showing correlation between log $K_D$ and volume (µl).](image)

- HPLC
- CE
- Dialysis
- Surface plasmon resonance
- Fluorescence anisotropy/polarization
- Isothermal titration calorimetry
- AlphaScreen
- Fluorescence intensity
- Gel electrophoresis
- Filter binding