

1 Is less more? Lessons from aptamer selection strategies

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15 1. Introduction. Antibodies versus aptamers

16

17 Biomarkers always have been in the focus of diagnostics and therapeutics, and their
18 exploitation in clinical trials and medical practice is steadily increasing. Although previous
19 research activities focused on nucleic acid biomarkers, which led to the development and wide
20 application of platforms for high-throughput analysis of DNA variants and mRNA expression
21 profiles, it has been recognized that analysis of protein biomarkers provides larger amount of
22 relevant information. Progress of proteomics technologies has brought about the explosion of
23 our knowledge in the field of disease-related protein patterns, and thousands of proteins have
24 been documented as biomarker candidates [1]. Thus, importance of selective detection and
25 targeting of individual proteins can hardly be overestimated. Presently, the antibody-based
26 assays are the most sensitive, specific and selective methodologies for detection and

27 characterization of proteins. Consequently, public domain initiatives have been launched to
28 deposit antibodies against all human proteins in databases with free accessibility (e.g. HUPO
29 Antibody Initiative) [2].

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

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

50 Figure 1.

51

52 While the best dissociation constants of published aptamer–target complexes seem to
53 be similar to those of antibody–antigens, aptamers are superior to antibodies in several aspects
54 [9]. These advantages of aptamers can be attributed to their chemical properties and *in vitro*
55 selection, and chemical synthesis. Oligonucleotides are conveniently prepared with high
56 reproducibility and purity; therefore, no batch-to-batch variation is expected in aptamer
57 production. Moreover, they withstand long-term storage at ambient temperature while
58 preserving their functionality, which can be tailored on demand during chemical synthesis, e.g.,
59 to aid their immobilization, to impart signaling properties, and/or to increase their resistance to
60 enzymatic degradation. Finally, the low immunogenicity and small size of aptamers are
61 appealing advantages with respect of their therapeutic and diagnostic application. Although all
62 these properties contribute to the growing popularity of aptamers, their *in vitro* selection could
63 probably be highlighted as their most important strength.

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

76

77

78 **2. Aptamer selection**

79 *2.1 Basic principles*

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

96 The first studies on aptamers involved mostly RNAs, motivated mainly by the
97 assumption that RNA can form more diverse 3D structures than DNA, which is believed to be
98 beneficial in terms of establishing a higher affinity to the target. However, the RNA SELEX is
99 more complex than the DNA SELEX [14] owing to the fact that additional *in vitro* transcription
100 steps are needed before and after each PCR amplification. Additionally, the RNA molecules

101 are prone to enzymatic degradation, which is a major problem to be addressed in most
102 applications. The authors of this review are not aware of any systematic study indicating a
103 higher affinity of either type of aptamers. The fact that both RNA and DNA aptamers are
104 frequently reported to form complexes of submicromolar or even subnanomolar dissociation
105 constants with their ligands further challenges the assumption of a marked difference between
106 their affinities. Beside the natural nucleic acids, RNA and DNA libraries containing various
107 modified nucleotides were also used for generating aptamers. Although the primary motivation
108 of these efforts was to increase the nuclease resistance of oligonucleotides, several
109 modifications also conferred aptamers with higher affinity [15]. Recent innovations have added
110 functional groups that mimic amino acid side-chains to expand the chemical diversity of
111 aptamers [16, 17]. These latest developments have eliminated one of the drawbacks of
112 conventional aptamers, namely the lack of hydrophobic moieties. This resulted in drastically
113 increased success rate of selection and yielded aptamers with subpicomolar affinity. Of note,
114 both publications have reported application of modified deoxynucleotides prognosticating the
115 dominance of DNA aptamers in the future.

116 Figure 2.

117
118 Implementation of aptamer production is much more complex than its simple, theoretical
119 scheme (Figure 2) would suggest, and the success of the procedure mainly relies on seemingly
120 minor experimental details of the selection. Consequently, following the introduction of
121 SELEX, numerous alternative approaches have been explored [18] with the general intention
122 of increasing the success rate, but also ensuring high speed [19, 20], low handled volumes [21],
123 minimal contamination and automation [22].

124 The conventional SELEX procedure needs high purity targets to ensure the selectivity
125 of isolated aptamers. In the case of proteins, this condition is generally fulfilled by using
126 recombinant proteins with various fusion tags (e.g. polyhistidine and glutathione S-transferase
127 (GST)). The fusion tags do not only simplify the purification protocol from the protein
128 overexpressing cell culture or *in vitro* translation system, but they also enable oriented
129 immobilization of the targets during the SELEX process; thus, the desired epitope of the protein
130 could be readily exposed for aptamer generation.

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

143

144 2.2 *Increasing the selectivity*

145 One of the first classical aptamer publications has already demonstrated that the basic
146 selection method could provide aptamers, which could discriminate among organic dyes with
147 very similar chemical structures [4]. Since then, a panel of modifications have been made to the

148 original protocol to increase further the selectivity of generated aptamers. The first
149 improvement has been described in the publication that presented the selection of DNA
150 aptamers for the first time [11]. The authors followed their previous protocol used for the
151 isolation of organic dye selective RNA aptamers, but when the pools that had been selected for
152 three cycles were applied to non-cognate dye columns, the ssDNA pools bound to every tested
153 dye, i.e., no selectivity was observed. Apparently, the oligonucleotides were nonspecifically
154 retained, either because of binding to the agarose matrix or universal dye binding. To remove
155 nonspecifically binding sequences, negative selection has been introduced, that is the selected
156 ssDNA pools of third cycle were flown over the non-cognate dye modified columns prior to
157 next positive selection cycle, which resulted in the removal of the sequences showing cross-
158 selectivity from the selection library. This simple negative selection cycle significantly
159 increased enrichment of selectively binding oligomers, and has been routinely applied during
160 aptamer selection since its introduction.

161 Soundness of this rationale was further validated by production of an RNA aptamer that
162 binds theophylline with 10,000-fold greater affinity than caffeine, which differs from the target
163 molecule only by an extra methyl group [8]. The aptamers were isolated by addition of the RNA
164 pool to theophylline coupled Sepharose column and the stringency of selection was increased
165 by removing of non-specific binders by washing the column with caffeine before collection of
166 theophylline selective oligonucleotides. This modified version of negative selection was
167 designated counter SELEX. Another outstanding example of discriminating capacity of
168 aptamers was also demonstrated by using negative selection combined with harsh washing
169 conditions to isolate arginine specific oligonucleotides [9]. The protocol involved a counter
170 selection with citrulline, but to increase the stringency of competition between free citrulline
171 and immobilized arginine, the column bound RNA was heat denatured and renatured in the
172 presence of citrulline before elution with arginine. This rigorous selection scheme led to a tight

173 binding RNA aptamer, which discriminates 12,000-fold between the D- and L-enantiomers of
174 arginine. It should be noted that confusingly, the negative and counter selections have been
175 widely used as synonymous expressions in the aptamer related publications.

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

186 The SELEX most often involves utilization of recombinant proteins, and this could lead
187 to limited applicability of produced aptamers. Majority of eukaryotic proteins are post-
188 translationally modified and many of them are membrane integrated thus the proteins in their
189 native conditions are often differently structured from the recombinant variants. Due to the
190 discriminating capacity of aptamers, using the standard, one ligand SELEX, even a slight
191 difference of native and recombinant proteins may preclude identification of aptamers, which
192 maintain their functionality with their physiological targets. This shortcoming of SELEX has
193 been illustrated with isolation of E-selectin specific thioaptamers [24]. Amongst the 14
194 aptamers selected by using recombinant protein only one bound to endothelial cells expressing
195 E-selectin, even though the applied, human recombinant protein had been obtained from
196 mammalian system. This observation highlights that integration of biologically relevant
197 conditions into the screening process increases the success rate of identification of aptamers

198 with pertinent biological activity. In the last decade, the Cell-SELEX has become a routinely
199 applied method; therefore, alternation of recombinant proteins and target protein expressing
200 cells during the steps of selection procedure can be expected to become a more widely applied
201 aptamer producing approach.

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

212

213 2.3 Selection without target immobilization

214

215 Improvements of the solid supports to minimize oligonucleotide absorption represent an
216 important aspect in the development of SELEX variants. In any case, additional stringent
217 counter-selection steps are needed to screen out those oligonucleotides that bind to the support.
218 Immobilization of the target is also critical in terms of having exposed the desired epitope for
219 aptamer generation. Therefore, from the plethora of alternative selection methodologies, the
220 homogeneous approaches need to be highlighted owing to their advantage of not requiring
221 target immobilization and, consequently, a solid support. These techniques are dominated by

222 electrophoretic methods, most notably by capillary electrophoresis [26] and free-flow
223 electrophoresis [27].

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

241 To alleviate the PCR bias issue of aptamer selection procedure, a target immobilizing
242 approach without iterative amplification cycles also has been developed [32]. MonoLex method
243 relies on application of affinity capillary column coated with the selection target and physical
244 segmentation of the column into slices following the chromatography of oligonucleotide
245 library. The different column fragment bound aptamer candidates are separately amplified with
246 a single PCR and their binding specificity is assessed by dot blot assay.

247 Although CE-SELEX and non-SELEX have been proved to be fast and effective ways
248 of isolation of protein selective aptamers, application of these methods also have their own
249 limitations. Since negative selection is not involved in KCE-based aptamer production, great
250 purity of target protein is a basic requirement of successful identification of aptamers that are
251 selective for the protein of interest. Thus, protein sample has to be thoroughly analyzed prior to
252 its application. The CE-SELEX and non-SELEX protocols can be accomplished in a week;
253 however, the optimal conditions of partitioning have to be determined individually for each
254 protein, which could be a challenging task. Furthermore, thermal band broadening of CE due
255 to Joule heating restricts the applicable ion concentration of partitioning buffers [33]; hence,
256 the selection conditions might not be adjustable to the circumstances of prospective usage of
257 aptamers [34]. Finally, one of the benefits of CE-SELEX, i.e., the small analyte requirement is
258 accompanied with an inherent shortcoming of the approach. The typical sample injection
259 volume in the range of nanolitres limits the sequence space that can be screened for target
260 binding. This is contrary to the optimal selection conditions whereas oligonucleotides are added
261 in large excess over the target molecule so that the probability of the presence of high-affinity
262 aptamers is increased, and competition for target proteins facilitates isolation of the best binders
263 from the pool.

264 Some of the above mentioned disadvantages of CE-SELEX such as sample volume
265 limitation and selection buffer restrictions may be overcome by using the free-flow
266 electrophoresis (FFE) technique in which the electrophoretic separation is performed on a
267 continuous flow of analyte in a planar flow channel. In contrast to CE where the electric field
268 is applied in the direction of the fluid movement, in FFE, the electric field is applied
269 perpendicularly to the pressure-driven flow to deflect the analytes laterally according to their
270 mobility [35]. Aptamers with low nM dissociation constants for protein targets were detected
271 following a single round of selection with micro FFE [24]. The electrophoresis techniques have

272 driven an obvious progress in terms of reducing the selection time; however, apparently there
273 is no significant improvement in lowering the dissociation constants of the selected aptamers
274 as compared with conventional SELEX techniques. For instance, dissociation constants of the
275 aptamers selected for IgE using the conventional SELEX method were as low as 10 nM [36],
276 somewhat lower than those of aptamers obtained by CE-SELEX (~ 40 nM [23]) and by micro
277 FFE (~ 20 nM) [24].

278

279 *2.4 Miniaturization of selection*

280 In most of the traditional SELEX procedures, non-selective oligonucleotides are
281 removed from target molecules either via membrane filtration or column chromatography, or
282 binding of the target protein to the wells of microtiter plates [15]. Due to the low partitioning
283 efficiency of these separation methods and the binding of oligonucleotides onto the matrix of
284 stationary phases, isolation of high-affinity, selective aptamers requires typically 8-15
285 cumbersome selection cycles. A significant improvement has been made to the conventional
286 selection technology with introduction of paramagnetic beads for target protein immobilization
287 [22]. Paramagnetic beads offer advantages over column chromatography in their ease of use
288 even in the microliter range. Hence, very small amounts of target protein coated beads can be
289 rapidly partitioned, stringently washed, and the protein bound oligonucleotides can be
290 subsequently eluted. These benefits of paramagnetic beads have made the manual aptamer
291 selection faster, more straightforward, and provided DNA and RNA aptamers with high affinity
292 [37, 38]. Significantly, an automated aptamer selection process has also been established by
293 using paramagnetic beads [22]. The enhanced, fully integrated robotic system accommodates
294 all steps of the aptamer production including isolation and amplification of selective RNAs.
295 The reported workstation can carry out eight selections simultaneously and can complete 12

296 rounds of selection in two days [39]. The same research group improved the protocol even
297 further by completing the system with *in vitro* transcription and translation of target proteins
298 [40]. *In vitro* translation is an effective way of high-throughput production of proteins thus
299 could serve as a supply of target proteins for aptamer selection [41]. Although these results
300 could make one envision a fully automated pipeline of aptamer production from coding gene to
301 protein-selective aptamer, the practical, high-throughput application of the combined system
302 has not been published, yet.

303 A mathematical model describing the optimal conditions for SELEX has pointed out
304 that strong competitive binding of oligonucleotides can yield the highest affinity aptamers [42].
305 To achieve the theoretically ideal ssDNA ratio, single microbead SELEX has been developed
306 and applied successfully for isolation of botulin neurotoxin selective aptamers. However,
307 manipulation of microscopic amount of beads demands delicate handling, thus it is not suitable
308 for routine application [43]. The advanced microfluidics provide miniaturized sorting
309 technologies for manipulation of individual particles or cells with continuous operation [44].
310 Realizing the benefit of these systems, a chip-based magnetic bead-assisted SELEX with
311 microfluidics technology, so called magnetic SELEX (M-SELEX) has been invented [45].
312 Partitioning efficiency (PE) is a generally accepted indicator of the success of separation. Lou
313 et al. have demonstrated that the PE of their continuous-flow magnetic activated chip-based
314 separation (CMACS) device is ca. 10^6 , thus it significantly exceeds the efficiency of
315 conventional separation methods, and is comparable to that of CE. They combined the
316 outstanding PE of CMACS device with usage of carboxylic acid activated paramagnetic beads
317 for target protein immobilization to reduce the nonspecific binding of negatively charged
318 oligonucleotides onto the beads. The effective separation and low background binding of
319 oligonucleotide library enabled isolation of Botulinum neurotoxin specific aptamer with low-
320 nanomolar dissociation constant after a single round of selection. However, the use of the

321 CMACS needed scrupulous tuning of the device with microscopy to achieve the high PE and
322 recovery of bead-bound oligonucleotides. To address this shortcoming, the research group
323 converted the CMACS device into micromagnetic separation (MMS) chip, which is more
324 robust and does not require a microscope for practical application [46]. Using the MMS chip,
325 they optimized their previous CMACS-based protocol by determining the ideal buffer flowing
326 rate, elevating the temperature of selection, and introducing a counter selection step.

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

339 Emerging of M-SELEX approach initiated mathematical remodeling of aptamer
340 selection procedure and the obtained numerical data highlighted a further advantage of MMS
341 chip-based method [48]. The authors compared the conventional filter-based SELEX and M-
342 SELEX and their calculations have drawn the attention again to the importance of the non-
343 specific, background binding of oligonucleotides onto the matrix of the stationary phase of the
344 process. According to the proposed model, the fraction of high affinity aptamers reaches 100
345 % at the 8th selection cycle with the low background binding M-SELEX, while the application

346 of filter for separation yields merely 12 % of high quality aptamers at the same round of
347 selection. The reduced number of iterative steps apparently implies faster aptamer producing
348 procedure, but more importantly, it also drastically decreases the enrichment of non-target
349 selective oligonucleotides resulting from intrinsic differences in the amplification efficiency of
350 nucleic acid templates.

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

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

366

367

Table 1

368

369 **3. Characterization of aptamer candidates**

370

371 Although the success of aptamer production is mainly dictated by the careful planning,
372 meticulous implementation and following of progression of selection [50], there is another
373 remarkable aspect of SELEX receiving little attention from the end users of aptamers. Since
374 most papers feature only a single aptamer, there is little awareness that the selection process
375 generally results in a large number of sequences. Ideally, all selected oligonucleotides need to
376 be evaluated individually in terms of their target binding properties to designate the most
377 auspicious aptamer candidates. Actually, this characterization is one of the most costly and
378 time-consuming tasks of the aptamer production. The sheer number of methods that have been
379 used to determine the dissociation constant of aptamer-target molecule complexes speaks both
380 the importance and difficulty of these measurements. The developed methods range from the
381 low-cost, simple approaches such as dialysis and filter binding assays to surface plasmon
382 resonance (SPR) and amplified luminescent proximity homogenous assay (ALPHA) requiring
383 dedicated instrumentation[27, 51]. As Figure 3 shows, the applied methodologies have different
384 sensitivities and requirements in terms of estimated analysis time and sample volume. The
385 measurements are further complicated since post-selection labeling or immobilization of
386 aptamers may significantly affect their binding distorting the K_D of native aptamer.
387 Additionally, the K_D values obtained from different methods could be noticeably divergent [52]
388 [53]. Considering all of these factors, K_D values should be determined with applying a method
389 that most closely simulates the circumstances in which the aptamer is intended to be used.
390 Noteworthy messages of these hindrances are that affinity of aptamers is suggested to be
391 measured by two different approaches and even the most carefully determined K_D values have
392 to be handled cautiously. Altogether, the practical value of aptamers cannot be revealed without
393 their thorough evaluation in the proposed application.

394

Figure 3.

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

419 for analytical studies, we have witnessed a tremendous development in the aptamer-based
420 analytical methodologies in the last decade. Most of the routine immunoanalytical
421 methodologies were seamlessly adapted to detect aptamer–ligand interactions [55]. Thus,
422 utilization of aptamers in label-free techniques such as SPR [56], SPR imaging [57], quartz
423 crystal microbalance [58, 59], microelectromechanical sensors [60], nano field effect transistors
424 (nanoFETs) [61], and electrochemical impedance spectroscopy [62], as well as in various
425 amplification schemes based on enzymes [63], luminescence-generating labels, and
426 nanoparticles [64, 65] have been demonstrated. Moreover, the range of bioassay methodologies
427 was further extended by exploiting the inherent properties of nucleic acid aptamers in molecular
428 beacons [66-68], ligation assays [69], electrophoresis [70], microarrays [71], and direct
429 reporting through the use of catalytic oligonucleotides (ribozymes and deoxyribozymes) [72].

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

434

435

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635

Table 1. Representative aptamers of various selection procedures.

	Selection method	Target	Selection rounds	K _D	Characterization method	Ref.
Immobilized target	Conventional SELEX	Cibacron Blue 3GA	7	100 μM	isocratic elution	5
		T4 DNA polymerase	4	5 nM	filter binding assay	6
	SELEX with modified nucleic acid	Keratinocyte growth factor	8	0.3-3 pM	filter binding assay	15
		Camptothecin derivative	9	86 nM	surface plasmon resonance (SPR)	17
	Cell-SELEX with modified nucleic acid	E-selectin	10	47 nM	electrophoretic mobility shift assay	24
		negative	Thrombin	5	25-200 nM	filter binding assay
	Counter	Theophylline	8	100 nM	equilibrium filtration	11
		L-arginine	20	330 nM	equilibrium dialysis	12
	Toggle	Human thrombin	13	2.8 ± 0.7 nM	filter binding assay	25
		Porcin thrombin		83 ± 3 pM		
	Monolex	Vaccinia virus	1	not available	fluorescence correlation spectroscopy SPR	32
	SELEX with magnetic separation	Thyroid transcription factor 1	15	3.36 nM	SPR	38
	Automated SELEX	Lysosome	12	31 nM	filter binding assay	39
		U1A protein	18	4.5 nM	filter binding assay	40
	FluMag-SELEX	Ibuprofen	10	1.5–5.2 μM	equilibrium filtration	13
Streptavidin		13	56.7 ± 8.2 nM	fluorescence binding assay	37	
Single microbead SELEX	Botulinum neurotoxin	2	3 nM	fluorescence polarization	39	
SELEX without immobilization	Free flow SELEX	IgE	4	29 ± 15 nM	fluorescence polarization affinity capillary electrophoresis	27
			4	58 ± 55 nM		
	CE-SELEX	IgE	2	40 nM	affinity capillary electrophoresis	26
			4	180 ± 70 pM	affinity capillary electrophoresis	29
			10	140 nM	SPR	30
Non-Selex	h-Ras protein	3	300 nM	non-equilibrium capillary electrophoresis of equilibrium mixtures	31	
Microfluidic selection	M-SELEX	Botulinum neurotoxin type A	1	33 ± 8 nM	fluorescence binding assay	45
		Transcription factor IIB	7	4 nM	electrophoretic mobility shift assay	19
		C-reactive protein	5	3.51 nM	SPR	20
	Selection with micromagnetic separation chip	Streptavidin	3	25-65 nM	fluorescence binding assay	46
		Platelet-Derived Growth Factor-BB	3	0.028 nM	fluorescence binding assay	47

639 Figure Captions

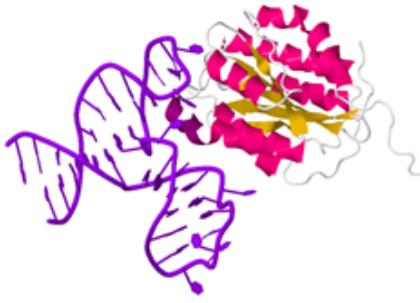
640 **Figure 1.** Elaborate 3D structures of aptamers. Crystal and solution structure of von
641 Willebrand factor [7] and the malachite green [8] binding aptamers A and B, respectively.

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

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

Figure 1

A



B

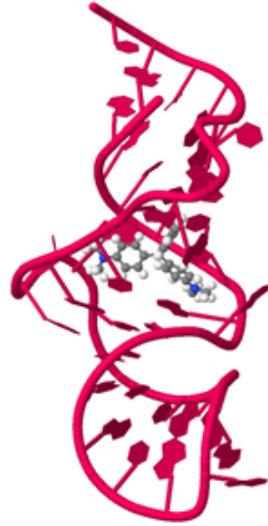


Figure 2

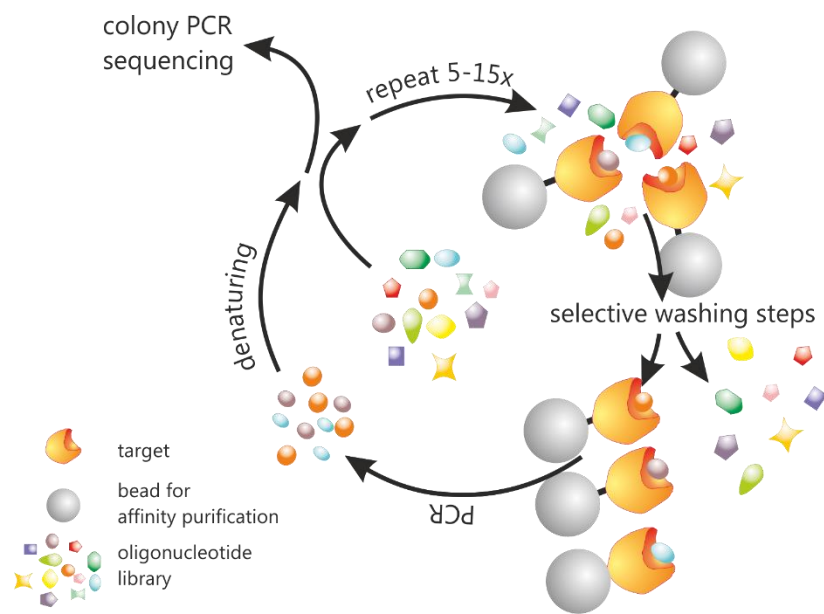


Figure 3

