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Research review paper

Current approaches in SELEX: An update to aptamer selection technology

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ABSTRACT

Systematic evolution of ligands by exponential enrichment (SELEX) is a well-established and efficient technology for the generation of oligonucleotides with a high target affinity. These SELEX-derived single stranded DNA and RNA molecules, called aptamers, were selected against various targets, such as proteins, cells, microorganisms, chemical compounds *etc.* They have a great potential in the use as novel antibodies, in cancer theragnostics and in biomedical research. Vast interest in aptamers stimulated continuous development of SELEX, which underwent numerous modifications since its first application in 1990. Novel modifications made the selection process more efficient, cost-effective and significantly less time-consuming. This article brings a comprehensive and up-to-date review of recent advances in SELEX methods and pinpoints advantages, main obstacles and limitations. The post-SELEX strategies and examples of application are also briefly outlined in this review.

Contents

1. Introduction

Aptamers are short synthetic ribo- and deoxyribonucleic acids that were first described in the early 1990s (Ellington and Szostak, 1990;

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Tuerk and Gold, 1990) and quickly became well-developed tools for various applications in research, diagnosis and therapy. The name "aptamer" comes from the Greek *aptus*, *i.e.* "to fit", and *merus*, *i.e.* "particle". In the first experiments, an RNA library was used as the starting oligonucleotide pool, resulting in generation of RNA aptamers. Later, single-stranded DNA (ssDNA) was also used for the generation of high-affinity aptamers. DNA is more stable than RNA, which makes the selection process easier. On the other hand, RNA may create a wider set of 3-D structures and can be synthesized inside the cells. Currently, both RNA and DNA libraries are used equally for generation of new aptamers. A third group of aptamers represented by short peptide sequences has also been described (Hoppe-Seyler et al., 2001).

Recently, a novel class of chemically derived nucleic acids, called xeno nucleic acids (XNAs) has emerged (Herdewijn and Marlière, 2009). These are oligonucleotides with an unnatural sugar backbone that were shown to possess similar features as DNA and RNA. XNAs can be replicated *in vitro* with specially mutated polymerases and store information in the manner of natural polynucleotides (Pinheiro et al., 2012). Also aptamers created of XNAs were generated successfully (Pinheiro et al., 2012) that opened new horizons for aptamers with advanced properties. For example, alterations of the phosphate-sugar backbone might lead to increased bioavailability and pharmacokinetics of aptamers, which is promising for therapeutic applications.

Almost all aptamers selected through SELEX (systematic evolution of ligands by exponential enrichment) exhibit high affinity to targets with a dissociation constant (K_d) in micromolar to nanomolar range. Therefore, aptamers are also called "chemical antibodies" (Fig. 1). Due to the lack of immunogenicity, aptamers are superior to antibodies and are attractive tools for various applications originally developed for antibodies (Jayasena, 1999). Until now, several aptamers have been involved in clinical trials. For instance, Pegaptanib (Macugen), marketed by Pfizer, is used in therapy of age-related macular degeneration (AMD) as an RNA aptamer selected against vascular epidermal growth factor (VEGF) (Ng et al., 2006) thereby preventing neovascularization during AMD (Ng and Adamis, 2006). Besides clinical therapy, aptamers are used in many other applications; such as detection of acute viral and bacterial infections (Kiilerich-Pedersen et al., 2013; Rotherham et al., 2012), detection of cancer biomarkers (Chang et al., 2013), detection of proteins in Western blot (Shin et al., 2010), as *in vivo* imaging agents (Hong et al., 2011), as capture ligands in chromatography (Zhao et al., 2008), surface plasmon resonance (SPR) assays (Chen et al., 2014), microarrays (Jung et al., 2013; Sosic et al., 2013), biosensors (Q. Wang et al., 2014).

One of the most promising applications of aptamers is targeted drug delivery. Used as specific molecules for targeting epitopes on cancer cells, aptamers designate specificity part of such delivery systems called aptamer-mediated nanovehicles (AMNVs). Another part of a nanovehicle is a chemotherapeutic drug bound to a nanocarrier. AMNVs consist of various nanocarriers such as the following: nanoparticles, quantum dots, liposomes, carbon tubes were published (L. Li et al., 2014; Savla et al., 2011; Xing et al., 2013; Zhang et al., 2014). Some of these AMNVs revealed increased in many times rate of cytotoxicity in comparison to drug by itself due to efficient drug delivery (Dhar et al., 2008, Kolishetti et al., 2010). These data provide a strong support for aptamer use in therapeutics delivery. The high selectivity and affinity combined with a low immunogenicity make aptamers preferable also to such delivery systems as the *in vitro* assembled virus like particles (Voráčková et al., 2014) despite the fact that the latter can be prepared in a high titre (Ulbrich et al., 2006) and the modification should result in efficient exposure of the modifying peptides linked to the N-terminus of the capsid protein as documented by detailed structural characterization of the particles (Bharat et al., 2012).

Since the first application of SELEX about twenty years ago, this method has undergone countless changes and improvements. Today, selection of a new aptamer takes hours instead of the weeks required for conventional SELEX. Novel approaches supported with computational software enable predictions of an aptamer structure and binding affinity even before this aptamer is selected, making the selection process much more cost-effective. Different chemical modifications introduced into aptamer sequences post-SELEX increase their stability and open new opportunities for the application of aptamers.

In this review, we bring a comprehensive description of some of the most well-established and widely used SELEX methods. Moreover, a brief description of post-SELEX modifications and aptamer databases is also included.

2. Conventional SELEX

SELEX was independently developed by two laboratories of Ellington and Szostak (1990) and Tuerk and Gold (1990) more than two decades ago. Since then, is has undergone numerous modifications and improvements. The conventional SELEX is a well-described technology (Tuerk and Gold, 1990). Generation of aptamers using this method lasts from few weeks up to a month. A chemically synthesized oligonucleotide library is usually used for aptamer selection. This library contains up to 60 oligonucleotides in random region, which are flanked by short constant regions, usually used to anneal primers during PCR. Several selection steps are introduced during conventional SELEX depending on the type of the desired aptamer. In the case of DNA aptamers, a chemically synthesized DNA library is incubated with target molecules. Unbound molecules are removed and the target/DNA complex is split. Released DNA sequences are amplified by PCR and additional round of selection is performed. Potential aptamers, obtained from a number of selection rounds, are sequenced and their binding kinetics are evaluated by various methods. In the RNA aptamer selection, additional steps of *in vitro* transcription and reverse transcription are introduced. Thus, a chemically synthesized DNA library is transcribed into a RNA library, which undergoes selection similar to DNA aptamers. Distinctly, RNA sequences released from their target are reversely transcribed into DNA and amplified by PCR. Multiplied DNA molecules are transcribed back into RNA and a new selection round starts over. Up

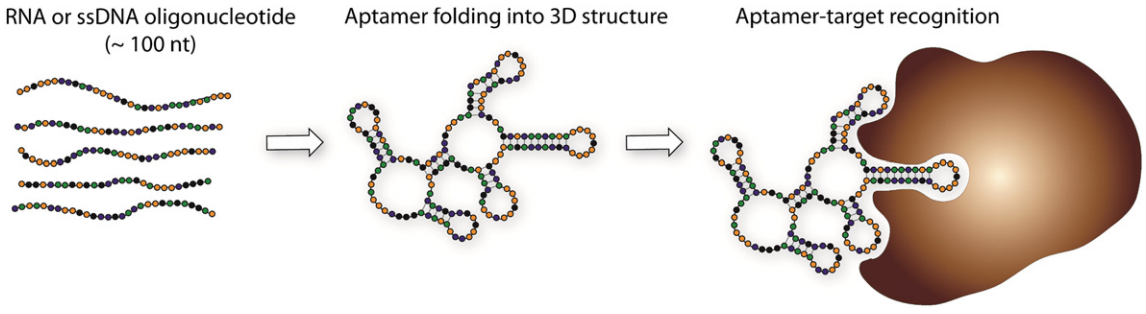

Fig. 1. Schematic representation of an aptamer–target interaction.
Modified according to Stoltenburg et al. (2007).

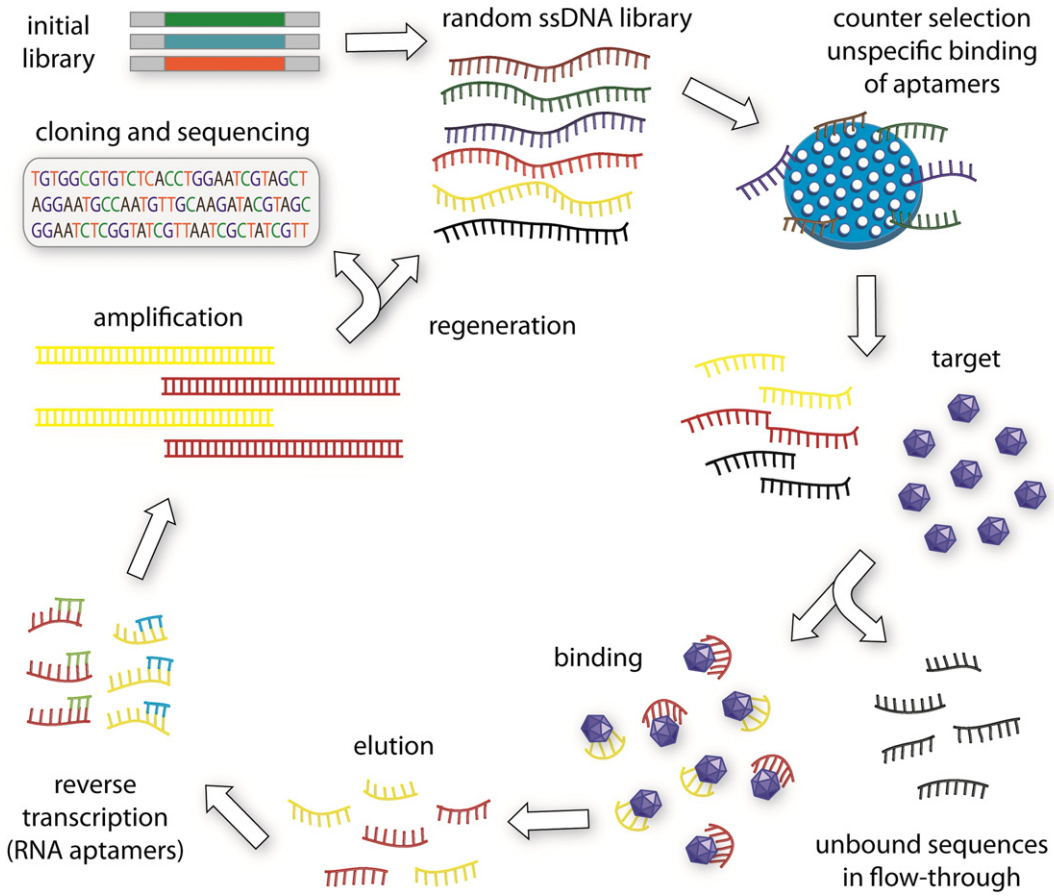

Fig. 2. The basic principle of aptamer generation by conventional SELEX. Selection starts from construction of an initial oligonucleotide library, which is directly proceeded to selection process. In many conventional SELEX protocols, the first step is a counter-selection against a matrix on which the target is immobilized. After removal of non-specifically bound aptamers, a pool of oligonucleotides is incubated with the target. Unbound sequences are removed; the target-bound oligonucleotides are collected, reverse transcribed into DNA (only for RNA aptamers) and forwarded to PCR amplification. After several selection rounds, cloning and sequencing steps are performed followed by evaluation of target affinity of the enriched aptamers.

to 20 rounds of selection are usually performed to enrich aptamers with a high target affinity (Fig. 2).

Conventional SELEX is a well-established and effective method but due to its large time- and labor-consumption, continuous development of alternative methods for aptamer selection has been inevitable.

3. Capillary electrophoresis-SELEX

One of the most frequently used variants of SELEX methods is CE-SELEX (capillary electrophoresis SELEX), first published in 2004 (Mendonsa and Bowser, 2004a). This method allows for reduction the number of rounds of aptamer selection from twenty, in conventional SELEX, to four in CE-SELEX while maintaining the affinity of an aptamer to its target (Mendonsa and Bowser, 2004a). The principle of this method is separation of an aptamer–target complex from unbound oligonucleotides according to their electrophoretic mobility (Fig. 3). The oligonucleotides bound to the target have a lower mobility than free oligonucleotides, which pass through a capillary into a waste. Then, pressure is applied to collect the aptamer/protein complex. Many aptamers selected by CE-SELEX have been published so far. They include the following: aptamers against IgE (Mendonsa and Bowser, 2004b), neuropeptide Y (ssDNA) (Mendonsa and Bowser, 2005), human immunodeficiency virus reverse transcriptase (HIV-1 RT, ssDNA aptamer) (Mosing et al., 2005), ricin toxin (ssRNA) (Tang et al., 2006), protein kinase C δ (Mallikaratchy et al., 2006) and anthrax protective antigen (ssDNA modified into nano aptasensor) (Cella et al., 2010).

Recently, a modification of CE-SELEX led to invention of micro free-flow electrophoresis (μFFE) (Jing and Bowser, 2011). This method brought a dramatic increase in aptamer partitioning efficiency in comparison to CE- and conventional SELEX, already after the first round of aptamer selection. The μFFE method allows continuous application, separation and collection of an oligonucleotide library performed in μFFE device. This leads to a higher yield of collected aptamers comparing to CE-SELEX, where only small volume of a sample can be applied. Another significant feature of μFFE is lower dilution of the oligonucleotide sequences after partitioning; 100-fold dilution of an injected sample in μFFE *versus* 6000-fold dilution in CE-SELEX. μFFE is more advantageous than the other types of CE-SELEX, but the necessity to fabricate a special μFFE device to perform electrophoresis strongly limits its applicability in an ordinary laboratory.

Other techniques based on capillary electrophoresis are kinetic capillary electrophoresis (KCE) methods of aptamer selection. These include NECEEM (non-equilibrium capillary electrophoresis of equilibrium mixtures) (Berezovski and Krylov, 2002), ECEEM (equilibrium capillary electrophoresis of equilibrium mixtures) (Drabovich et al., 2005), and SweepCE (sweeping capillary electrophoresis) (Okhonin et al., 2004). The common principle of these methods is partitioning of a mixture of DNA library molecules with target proteins using a gel-free capillary electrophoresis. NECEEM is a simple method with high efficiency of aptamer partitioning. An oligonucleotide library is incubated with a target molecule. When oligonucleotides and target complexes reach the equilibrium with free molecules, the mixture is injected into a capillary and separated by electric field. During the separation process, both unbound DNA and proteins migrate as a single electrophoretic zone, but DNA/protein complex dissociates slowly during migration creating nonequilibrium production of DNA and protein. Both, the

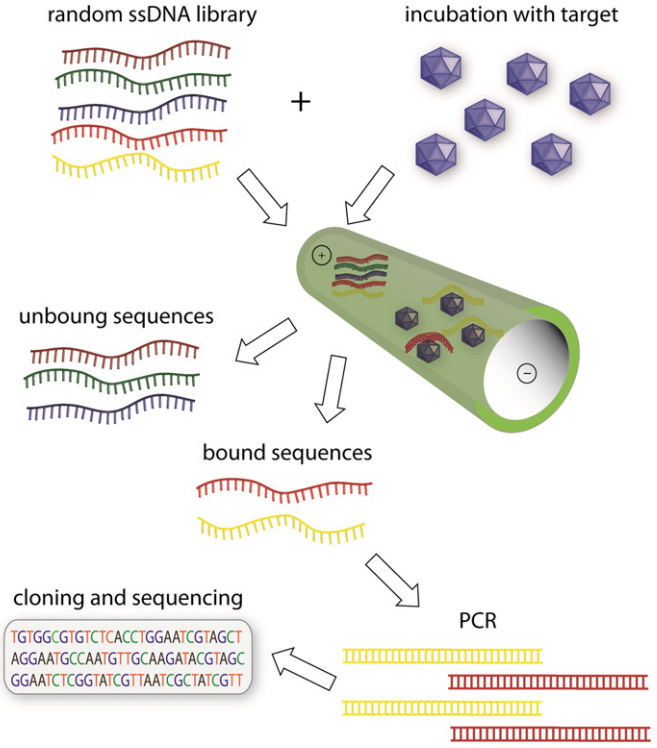

Fig. 3. Schematic illustration of capillary electrophoresis-SELEX technology. An initial pool of oligonucleotides is first incubated with the target, then the mixture is separated based on the mobility shift. The bound sequences are collected, amplified by PCR and used for cloning, sequencing and evaluation of affinity of obtained aptamers.

dissociation constant (K_d) and the off-rate constant (K_{off}) of an aptamer–target molecule complex can be calculated from an electrophoreogram.

Unlike NECEEM, in the ECEEM method the target molecule is present in the running buffer. This modification creates different partitioning conditions compared to NECEEM. During ECEEM the aptamer–target complex is maintained at equilibrium, helping to separate the complex from free oligonucleotides present in the applied library as well as to separate aptamers with different dissociation constants. ECEEM cannot be performed without preceding NECEEM, which defines overall affinity of an oligonucleotide library to the target molecule, K_d and an aptamer collection window. However, ECEEM enables creation of "smart aptamers" with predefined K_d, which can be extremely useful in research and development of "smart drugs" for clinical therapy.

Simultaneously with the aforementioned techniques, an alternative method SweepCE (sweeping capillary electrophoresis) was reported. The capillary prefilled with a DNA solution is used for electrophoresis of the target protein. Due to its faster electrophoretic mobility, the protein reaches DNA and forms the protein–DNA complex with a higher velocity than that of unbound DNA. This method provides not only K_d and K_{off} of the oligonucleotide–protein complex, but also a constant of the complex formation (K_{on}), which extends the knowledge of aptamer–target complex interaction (Okhonin et al., 2004). A more recent NECEEM-based non-SELEX method (Berezovski et al., 2006) has enabled generation of aptamers within only 1 h. No PCR amplification of oligonucleotide sequences is required during the partitioning, which is achieved by creation of an aptamer collection window during NECEEM. For this purpose, the bulk affinity of a DNA library to the target protein was estimated first. No detectable complex of DNA and protein was observed. The aptamer collection window was detected using the electrophoreogram of both a DNA library and pure protein. The left boundary of this window was assigned according to the position of pure protein peak, while the right boundary was assigned to a pure DNA peak. Three rounds of NEECEM partitioning were required to enhance the affinity of a DNA library to a target protein by at least four orders of magnitude higher than the initial bulk affinity of a DNA library to the target protein. Due to the fact that no amplification step is included between the partitioning rounds, all the DNA molecules in the enriched libraries had unique random sequences. Such abundance of affine DNA molecules can be helpful when choosing the most potent aptamers to a certain protein. A number of aptamers have been generated using this method, so far, such as aptamers to H-Ras protein (Berezovski et al., 2006), farnesyltransferase (Berezovski et al., 2006), signal transduction proteins such as Cdc42-GTP, p21-activated kinase 1 (PAK1), MRCK (myotonic dystrophy kinase-related Cdc42-binding kinase α) (Tok et al., 2010) and bovine catalase (Ashley et al., 2012).

4. Magnetic bead-based SELEX

A SELEX method employing magnetic beads for immobilization of the target was published in 1997 (Bruno, 1997). In this method, called magnetic bead-based SELEX, the target protein was immobilized on magnetic beads, incubated with an oligonucleotide library and resulted in a target–aptamer complex that was separated from unbound oligonucleotides by a magnetic separator (Fig. 4). Binding of an aptamer to its target was qualitatively evaluated by epifluorescence microscopy. This approach provided several DNA aptamers including those against 4-chloroaniline, 2,4,6-trichloroaniline and pentachlorophenol. Later on, flow cytometry was introduced for DNA quantification instead of fluorescence microscopy (Wang et al., 2009). A more convenient and effortless, FluMag-SELEX method was published in 2005 (Stoltenburg et al., 2005). The principle of FluMag-SELEX is based on immobilization of the target on magnetic beads. After the first round of selection, ssDNA was amplified by PCR and labeled using fluorescein-modified primers for quantification of selected aptamers in further selection rounds. The obtained oligonucleotides are then cloned and sequenced. The dissociation constants of the enriched aptamers can be determined by fluorescence measurement. Aptamers against streptavidin (Stoltenburg et al., 2005), ibuprofen (Kim et al., 2010) and polychlorinated biphenyls (Xu et al., 2012) were generated using this approach. Capture-SELEX is a novel promising method based on FluMag-SELEX (Stoltenburg et al., 2012). The major distinction of capture-SELEX is immobilization of the DNA library instead of the target on magnetic beads. Such modification enabled selection of aptamers against small targets that could not be immobilized. DNA quantification was performed by fluorescein labeling after each round of selection, similarly as in FluMag-SELEX. Aptamers against kanamycin A, sulfacarbamide, sulfamethoxazol and solatol hydrochloride were generated using capture-SELEX (Stoltenburg et al., 2012).

Research of magnetic utilization in SELEX has led to the development of microfluidic-based SELEX technology (Lou et al., 2009). For example, continuous-flow magnetic activated chip-based separation (CMACS), which contains micro fabricated nickel ferromagnetic structures, creates a magnetic field within the micro channel. The hydrodynamic and magnetophoretic forces were then applied for partitioning of aptamers to protein immobilized on magnetic beads. Such microfluidic-based methods for aptamer selection are called M-SELEX. The CMACS device performed well in high-throughput but was difficult to handle. Any obstacles in the micro channel caused bead aggregation resulting in low purity of aptamers reported later by the same group. A micro-magnetic device with improved separation was later developed by Oh et al. (2009). This micro-magnetic separation chip (MMS), which is easy to handle, enables large-scale isolation of aptamers. Various targets of aptamers generated by M-SELEX are listed as follows: BoNT/A-rLc protein (Lou et al., 2009), bovine serum albumin (Oh et al., 2009), apolipoprotein E3 (Ahmad et al., 2011), influenza A/H1N1 virus (Lai et al., 2014). M-SELEX proved to be a highly efficient strategy of producing aptamers with low K_d. More importantly, M-SELEX is an automated platform, which enables rapid generation of aptamers with high target affinity.

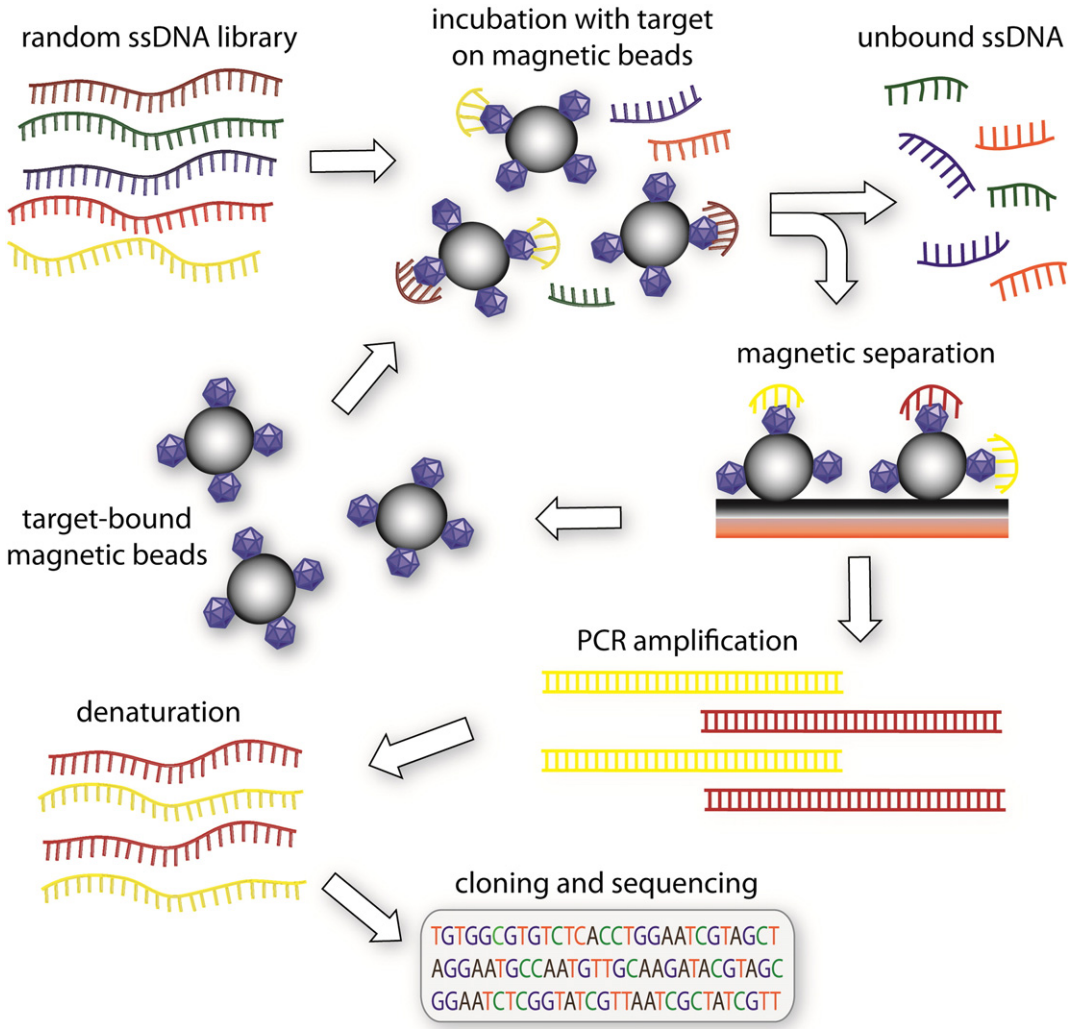

Fig. 4. Basic steps of magnetic bead-based SELEX. An initial library is incubated with magnetic beads coated with the target molecule. Then magnetic force is applied to separate oligonucleotides bound to the target from unbound sequences. Bound oligonucleotides are eluted, amplified by PCR and the cycle is repeated. After several selection rounds, aptamers are amplified, cloned and subjected to evaluation of their affinity.

Another method of aptamer generation based on M-SELEX and high-throughput DNA sequencing has been recently published by Cho et al. (2010). This quantitative selection of aptamers through sequencing (QSAS) includes three rounds of M-SELEX followed by high-throughput sequencing of the DNA enriched library, computational analysis and measurement of K_d by fluorescence binding assay. QSAS emerged as a highly efficient and rapid method for aptamer preparation.

5. Cell-SELEX

All previously described SELEX methods are based on knowledge of the target for aptamer selection. The only exception is cell-SELEX. This well-developed method does not require prior target knowledge. Whole cells, both eukaryotic and prokaryotic, can be used to generate highly specific aptamers using this technique. As in conventional SELEX, an oligonucleotide library is incubated with the targets, which are whole cells in the case of cell-SELEX. The unbound oligonucleotides are washed out and aptamers are separated from the target and amplified by PCR (Fig. 5). Cell-SELEX may require up to 35 selection rounds but most commonly 8–10 rounds are applied in order to obtain aptamers with high affinity. A series of aptamers against various cancer cell types were generated by this method. Some of them interact with cells derived from human hepatocarcinoma (Ninomiya et al., 2013), mouse tumor endothelial cells (Ara et al., 2012), glioblastoma multiform (Bayrac et al., 2011), small cell lung cancer (Kunii et al., 2011), lung adenocarcinoma (Jiménez et al., 2012), prostate cancer (Y. Wang et al., 2014) and colorectal cancer (W.M. Li et al., 2014). Liu et al. (2012) described also aptamers against normal mature adipocytes. A substantial number of aptamers was produced against bacterial cells, such as *Salmonella typhimurium* (Dwivedi et al., 2013), *Escherichia coli* (Kim et al., 2013), *Vibrio parahaemolyticus* (Duan et al., 2012), *Trypanosoma cruzi* (Nagarkatti et al., 2012) using cell-SELEX technology. Besides the above-mentioned, aptamers were also developed against baby hamster cell lines (BHK-21) infected by rabies virus. Such types of aptamers, capable to suppress viral replication (Liang et al., 2012), could have a great impact on medicine.

Another distinctive SELEX method using whole cells is a combination of cell-internalization SELEX with high-throughput screening. The Giangrande group in cooperation with Roche first published it in 2012 (W.H. Thiel et al., 2012). After each round (9 in total), all internalized RNA was isolated, transcribed into DNA and amplified by PCR. Concentrated salt solution was used to remove the unbound and non-internalized RNA. The obtained aptamers were analyzed by high-throughput sequencing and bioinformatics assays. Vascular smooth muscle cells are a prime example of a target for aptamers raised by cell-internalization SELEX combined with high-throughput screening (W.H. Thiel et al., 2012). Another case using cell-based selection

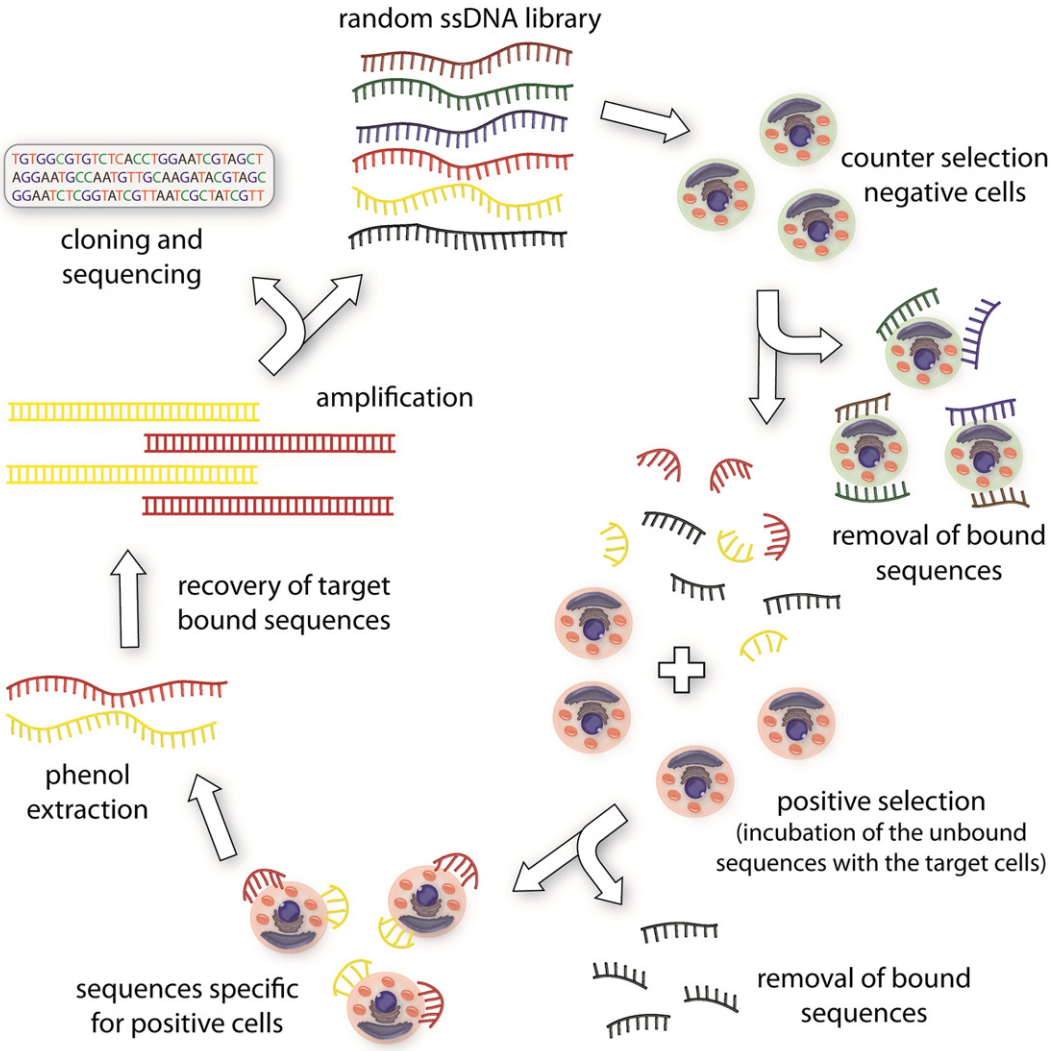

Fig. 5. Schematic representation of cell-SELEX. A chemically synthesized oligonucleotide library is incubated with cells that are not related to the target cells in a counter selection step. Unbound oligonucleotides are collected and incubated with cells of interest. Using phenol extraction, bound sequences are split from their targets, amplified by PCR and subjected to next selection round. After selection is completed, enriched aptamers are used in further steps of cloning, sequencing and target affinity evaluation.

approach enabled identification of RNA aptamers able to internalize into cells and target therapeutic siRNAs to HER-2/neu breast cancer cells (K.W. Thiel et al., 2012). Aptamers with efficient intracellular internalization are promising tools as carriers for therapeutic applications.

6. *In vivo* SELEX

In vivo SELEX is a technique that generates aptamers in living organisms. In the first *in vivo* SELEX experiment, an infectious pool of HIV-1 DNA genomes with random mutations was transfected into CD4 + T cells and the protocol proceeded with multiple rounds of viral replication (Berkhout and Klaver, 1993) followed by cloning and sequencing. This work led to selection of replication-competent viruses from the initial library. A similar approach was used later for identification of Rous sarcoma virus mutants by using randomized sequences (Doria-Rose and Vogt, 1998) and domains of the HIV-1 5′ leader RNA (van Bel et al., 2014).

Another variant of *in vivo* SELEX was developed to select aptamers capable of specific localization inside a tumor of a living organism (Mi et al., 2010). The overall selection process in this *in vivo* SELEX is very similar to conventional SELEX; with the exception of using live organism for the selection process instead of using isolated targets which are employed in conventional SELEX. Thus, Mi et al. (2010) used mice with intrahepatic colorectal metastases as a selection model of RNA aptamers for the *in vivo* SELEX. In detail, RNAs with 2-F′-pyrimidine substitutions were injected into mice and liver containing intrahepatic tumor that was isolated. RNA aptamers were extracted from the liver, amplified and injected into other mice bearing the same tumor. Fourteen selection rounds were performed in the same manner. As a result, aptamers with high affinity (low nanomolar range K_d) to p68 and RNA helicase were selected. Recently, aptamers capable of penetrating through the blood–brain barrier have been selected in wild-type mice employing *in vivo* SELEX (Cheng et al., 2013). The selection scheme resembles the above-described one, but after twelve rounds a negative selection step was introduced. This extra step was comprised of incubation of the RNAs obtained from the previous rounds with mouse serum. Further selection continued up to 22 rounds. *In situ* hybridization was then carried out in order to test the distribution of the obtained RNA aptamers. Their localization in cortex, hippocampus, cerebellum and striatum brain compartments was confirmed by using this approach (Cheng et al., 2013). These results indicate that *in vivo* SELEX can be used as a beneficial alternative to the *in vitro* SELEX as it may overcome some problems with tumor specificity of the *in vitro* obtained aptamers.

7. One-round SELEX

A series of studies were carried out to minimize the number of selection rounds in aptamer generation. As a result, different strategies for one-round aptamer selection were published: MonoLEX (Nitsche et al., 2007), NanoSelection (Peng et al., 2007), ASExp (Aptamer Selection Express) (Fan et al., 2008), selection on glass coverslips (Lauridsen et al., 2012) and elution by increasing salt concentration (Arnold et al., 2012). MonoLEX is an *in vitro* method for aptamer selection based on affinity chromatography that yielded potent aptamers against complete particles of a vaccinia virus (Nitsche et al., 2007). The principle lies in incubating a resin-bound target (*e.g.* heat inactivated viral particles) with a DNA library. The unbound DNA oligonucleotides are washed out, the column resin is cut into slices and the eluted aptamers are amplified by PCR. Further, aptamer-binding affinity to the target is assayed to select potent aptamers.

The second one-step aptamer selection method, NanoSelection, is based on incubation of beads carrying fluorescently labeled oligonucleotides with a protein target immobilized on a coverslip. After washing of the unbound oligonucleotides, aptamers bound to the target are detected by fluorescence microscopy and extracted by atomic force microscopy (AFM) in a contact mode. Finally, the extracted aptamers are amplified by PCR. NanoSelection showed good results and its high reproducibility has been proved by trial experiments, where a known aptamer sequence was extracted from an oligonucleotide pool eight consecutive times (Peng et al., 2007).

The ASExp method is based on the magnetic separation using microbeads. Thus, a ssDNA library is PCR amplified to obtain dsDNA oligonucleotides. These are then incubated with the target, which denatures double stranded molecules creating the ss-DNA/target complex. After the incubation, the reaction mixture is supplemented with magnetic beads bearing random ssDNA sequences interacting with ssDNA/target complex. Following magnetic separation leads to isolation of potential aptamers. ASExp methods provided aptamers generation against Ba spores, Shiga toxin, *Francisella tularensis* bacteria and botulinum neurotoxin (Fan et al., 2008, Kiel et al., 2008).

Similar principles of one-step aptamer generation has been applied in another recently published study (Lauridsen et al., 2012) where the target, α-bungarotoxin, was covalently linked to a pegylated glass coverslip, incubated with DNA oligonucleotide library and washed with an excess amount of buffer. Then, the fluorescent emission of the aptamer/target complex was monitored by fluorescence microscopy. The coverslips were crushed and heating eluted bound sequences. The selected aptamers were amplified by PCR, cloned and amplified in *E. coli*. By this procedure, anti-α-bungarotoxin DNA aptamers with K_d in low micromolar range were obtained (Lauridsen et al., 2012). Aptamer selection on glass coverslips does not require any special equipment, which makes this method more convenient than the other described one-step aptamer selection methods.

In the same year, an effortless one-round SELEX was developed by Arnold et al. (2012). Kalikreine-related peptidase 6 (KLK6) was immobilized in a 96-well ELISA (enzyme-linked immunosorbent assay) plate and incubated with the aptamer library overnight. Afterwards, unbound and nonspecifically bound aptamers were separated by salt solutions in a step elution gradient. The most tightly bound specific aptamers were eluted by the highest salt concentration. This fraction was collected

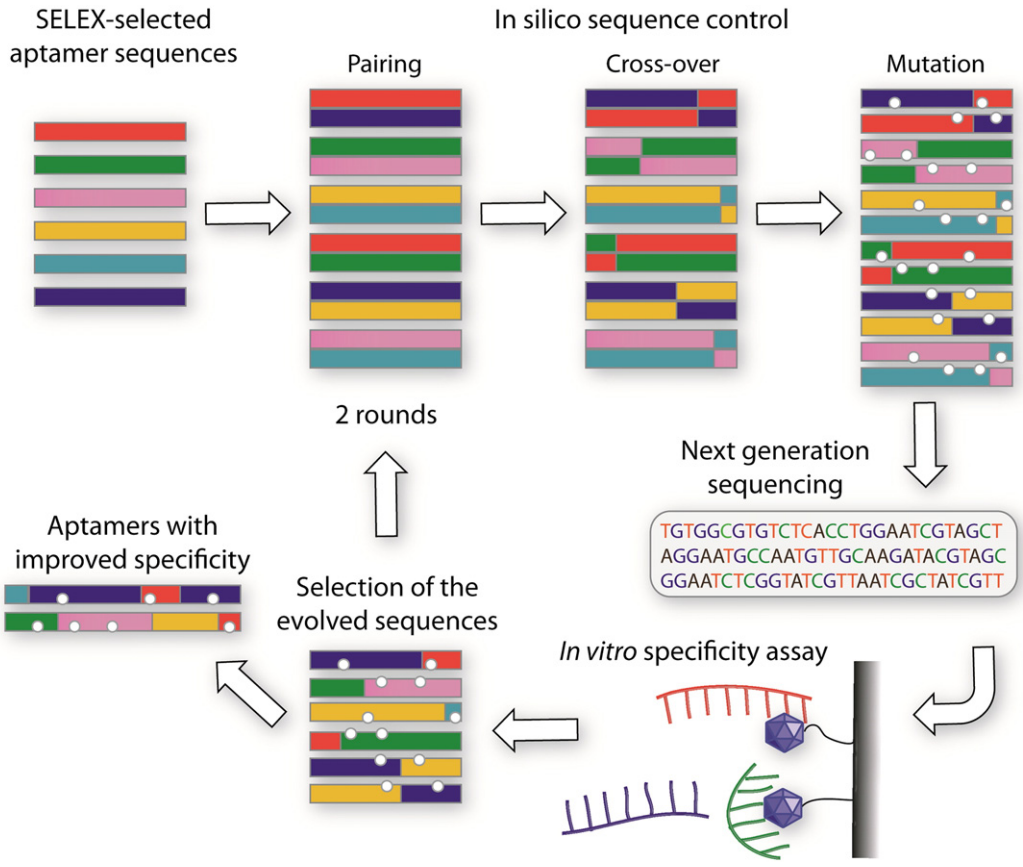

Fig. 6. The scheme of *in silico* maturation of aptamers. Aptamers that were derived by SELEX are evaluated for target specificity by an aptamer blotting assay. Sequences of selected aptamers are replicated *in silico* and paired randomly with other sequences, followed by crossing over and point two-base mutations. The specificity indexes of the new oligonucleotides are again evaluated and chosen aptamers undergo a second round of *in silico* maturation.
Modified according to Savory et al. (2013).

Table 1
A list of modified SELEX methods and their brief description.

Name of method (reference)	Principles of the method
Blended SELEX (Smith et al., 1995)	In this method, an RNA library is annealed to DNA that is covalently attached to a weak enzyme inhibitor. This modification enables an increase in the inhibition efficiency of the enzyme. Thus, a covalently modified library is incubated with the target protein. The aptamers are separated from unbound molecules by gel electrophoresis. RNA is extracted from the gel, transcribed by to cDNA reverse transcriptase and amplified by PCR. The procedure is repeated. As a result, selected aptamers with high target affinity stabilize interaction of the enzyme and the inhibitor and increase inhibition activity and specificity of the latter.
Cross-linking SELEX or PhotoSELEX (Golden et al., 2000; Jensen et al., 1995)	In this technique, light-sensitive nucleotides (*e.g.* 5-bromo-2′-deoxyuridine) are introduced into the initial oligonucleotide library. When excited by UV, these modified nucleotides form covalent bonds with aromatic and sulfur-containing amino acids of a target protein, which increases the binding affinity of aptamers to their targets. The PhotoSELEX protocol is as follows: an oligonucleotide pool containing modified nucleotides is incubated with the target and then the complex is cross-linked by UV light irradiation. Bound and unbound nucleotides are partitioned using SDS-PAGE. Bound oligonucleotides are then isolated from the gel, split from the target protein and subjected to PCR amplification. Then selection is repeated with enriched oligonucleotides. After several selections, aptamers are cloned and sequenced. This method exhibits greater potential in selection of aptamers with increased affinity and specificity to the target over other variants of SELEX.
Spiegelmer® Technology or mirror-image SELEX (Klussmann et al., 1996)	In this technology, aptamers against an enantiomer form of the target are selected through the standard SELEX protocol. After several selection rounds, enriched aptamers are sequenced. Obtained sequences are chemically synthesized using unnatural L-nucleotides that form an aptamer binding the native form of the target protein. Such L-oligonucleotides exhibit superior properties over aptamers consisting of natural oligonucleotides due to their increased stability to nuclease degradation.
Chimeric SELEX (Burke and Willis, 1998)	This approach uses two different pools of RNA oligonucleotides to obtain chimeric aptamer that can exhibit several features. Thus, aptamers against two distinct targets are first selected. These RNA aptamers are transcribed into cDNA and amplified by PCR. The obtained two cDNA pools are mixed, heated and annealed in primer binding regions. The ends of these regions are enzymatically extended to obtain chimeric dsDNA. This DNA is transcribed into RNA, which is again reselected to enrich the population, where the activities of both aptamers will be retained within one chimeric aptamer molecule.
Deconvolution SELEX (Morris et al., 1998)	In this method different aptamers selected for the various targets are separated from the complex mixture. Initially, ghosts of red blood cells were selected as a complex target. In this approach the standard SELEX protocol is applied first to enrich the aptamers against the complex target. Aptamers from the last round of selection are then cloned and the sequences of the obtained clones are determined. The sequences are then divided into families. Aptamers from each family are labeled with a photoreactive molecule at the 5′ end and radiolabeled at 3′ end. Such aptamers are incubated with the complex target. After aptamer binding, proteins of the target are visualized by SDS-PAGE and autoradiography. In this manner an aptamer that binds to a certain protein of a complex target is identified.
Multi-stage SELEX (Wu and Curran, 1999)	This method is a modified chimeric SELEX and was developed for the selection of allosteric synthetic DNA molecules. In the first stage, two different oligonucleotide libraries are incubated with two different targets resulting in selection of aptamers specific for a certain target. In the second stage, the counter selection is performed to remove the aptamers specific for the immobilization matrix. In the third stage, these two pools of aptamers are fused by PCR. This chimeric pool is then applied to six rounds of selection against the first target using affinity chromatography. Then the second target is applied to elution of enriched aptamers from the column. In the fourth stage, the enriched aptamers are again separated and used in a counter selection round. As a last step, the two DNA pools are again fused and carried through the third stage. Finally, the enriched aptamers are cloned and characterized.
Indirect selection (Kawakami et al., 2000)	This method generates metal ion-dependent aptamers interacting with their target only in the presence of a metal. For this standard SELEX protocol is applied in the presence of a metal salt. After the sixth and twelfth selection rounds, the aptamers are cloned and sequenced. SPR analysis is then applied to measure binding affinity of enriched aptamers. This method enables obtaining aptamers that would exhibit their function in living organism only in the presence of particular metals.
Crossover-SELEX (Hicke et al., 2001)	In crossover-SELEX both purified proteins and cells bearing the same protein on their surface are used as targets. The selection is performed in parallel with both targets. Aptamers incubated with a purified protein are directly cloned and analyzed, whereas enriched sequences from cell-SELEX are used for further incubation with the purified protein. After two selection rounds these aptamers are also cloned and sequenced. In crossover selection first are selected aptamers against target in its native state on the surface of the cells. The two additional rounds of selection with purified target enable enrichment of high-affinity aptamers that are rare in tumor cell aptamer pool.
Toggle-SELEX (White et al., 2001)	The technique is used to select aptamers with cross-reactivity to two homologous targets. In the first round, an oligonucleotide library is incubated with a mixture of these targets. Enriched oligonucleotides are used in the next selection rounds where they are incubated with targets separately. First, oligonucleotides are incubated with one target and then bound sequences are eluted. In the next round, these sequences are incubated with the second target. Bound sequences are again isolated and used in the next selection rounds. After thirteen selection rounds, the aptamers are cloned and sequenced. This method can be used to select aptamers with a broad specificity and improved *in vivo* functionality.
Truncation SELEX (Pagratis et al., 2001)	This method has several modifications where fixed sequences in oligonucleotide library are reduced or eliminated. In one of the methods, an oligonucleotide library containing fixed sequences is incubated with a target molecule. After binding, oligonucleotides are split from the target and amplified by PCR. Then, fixed regions of the selected sequences are replaced into new ones and the selection round is repeated. In another variant, a library consisting of random regions is incubated with a target and bound sequences are separated. Then they are hybridized with a library containing fixed regions and random regions complementary to the enriched sequences. Such hybridized sequences are amplified by PCR, fixed regions of amplified oligonucleotides are then removed and these oligonucleotides are forwarded to a new selection round. This technique reduces participation of fixed sequences in selection process.
Expression Cassette SELEX (Martell et al., 2002)	The approach uses aptamers selected through conventional SELEX for development of vectors bearing aptamer sequence. For example, Martell et al. inserted aptamer sequence into a tRNA expression cassette. The construct contained a promoter, tRNA sequence and the aptamer sequence. After transcription, a chimeric tRNA–aptamer complex retained inhibition activity of the aptamer and exhibited greater stability due to extended aptamer length. This method is useful for the expression of high levels of functional aptamers in mammalian cells.
Transcription free-SELEX (Smith and Gold, 2002)	In this method, a DNA template is incubated with a mixture of three RNA libraries that are as follows: two specific regions complementary to part of DNA template and one random RNA sequence. When hybridized to the DNA template, these three RNA regions are ligated together and used in selection steps. After aptamers are enriched, RNA is reverse transcribed into cDNA that serves as a template in next selection rounds. This technology is useful when using for selection of unnatural RNA that cannot be transcribed by RNA polymerase. This method is patented.
SPR-SELEX (Khati et al., 2003)	Surface plasmon resonance (SPR) assay enables not only the selection of aptamers but also monitoring of the interaction kinetics between an oligonucleotide library and a target protein. In this assay, the target is immobilized on SPR-chip and solution containing an oligonucleotide library is injected into a biosensor chip. The unbound sequences are washed out, the aptamers are eluted, amplified by PCR and whole round is repeated. At each selection round oligonucleotides are also injected into the chip without target as a counter selection step. The evaluation of aptamer enrichment is performed. It is based on a slow dissociation rate of aptamers from the target. Obtained aptamers exhibit K_d in a low nanomolar range.
Subtractive SELEX (Wang et al., 2003)	This method enables selection of aptamers distinguishing two closely related structures (*e.g.* differentiated from normal cells). During this procedure the oligonucleotide library is incubated with normal cells prior to each selection round, where differentiated cells are used as a target. A standard SELEX protocol is applied, followed by affinity evaluation of obtained aptamers. The method results in selected aptamers with high resolution of similar targets.

Table 1 (*continued*)

Name of method (reference)	Principles of the method
Tailored SELEX (Vater et al., 2003)	This method enables primer-free selection of aptamers. After each round of selection RNA, the library is ligated with primers, reversely transcribed into cDNA, amplified by PCR. The reverse DNA strand is then cleaved by alkaline fission at a special cleavage site. Such truncated reverse strands then serve as templates for transcription and repetition of the process. Aptamers selected through conventional SELEX often should be truncated after selection because their primer regions are involved in target domain binding. Whereas Tailored SELEX enables selection of aptamers without primer sequences and thus avoids post-SELEX truncations.
Conditional SELEX (Smith and Gold, 2004)	This method consists of several variants and is used for the selection of regulated aptamers. These methods enable selection of aptamers that either can bind with the target only in the presence of a regulator molecule, or they can bind to the target only if a regulator molecule is absent. In the first case, the library is incubated with a target in the presence of regulator molecule and bound oligonucleotides are collected. In the second one, the aptamers are incubated with the target in the absence of the regulator and then bound oligonucleotides are collected and incubated with the target in the presence of regulator. Here, only unbound sequences are collected, amplified by PCR and subjected the next selection rounds. These methods are patented.
On-chip selection (Asai et al., 2004)	This method utilizes microarray technology in combination with point mutations of selected sequences using a genetic algorithm. First, random sequences generated *in silico* are synthesized on CombiMatrix chip and incubated with the target. A sequence with the highest specificity is then chosen as a "mother". Then single and double mutations are introduced *in silico* to this sequence and the round repeats. The first-generation aptamer is immobilized on SPR chip and is used in binding studies with determination of K_d for aptamer/target complex. This method enables rapid and automated selection of aptamers against a large amount of targets.
TECS-SELEX (Ohuchi et al., 2006)	Target expressed on cell surface-SELEX (TECS-SELEX) utilizes cell-SELEX to select proteins ectopically expressed on the cell surface. In this protocol, rounds of positive selection against cells of interest alternate with counter selection rounds using cells that do not express the target protein. After eleven rounds of selection aptamers with nanomolar K_d are selected.
DeSELEX and Convergent selection (Layzer and Sullenger, 2007)	This method was first described for selection of RNA aptamers against a gamma-carboxyglutamic acid (GLA) protein mixture. GLA protein proteome from human plasma was incubated with a RNA library, RNA bound to this complex target was amplified and the process was repeated seven times. The aptamers from the last round were cloned and sequenced. Then, anticoagulant activity was determined by clotting assays. It was found that 80% of enriched aptamers bind to Prothrombin. The most prevalent clones among the aptamers were annealed with the primer intended to prevent folding of the aptamer sequence and binding to the target. This redirected the selection towards other less-abundant aptamers and proteins in a complex target because a standard complex selection did not generate aptamers against other less-abundant proteome targets. The convergent selection was then performed to determine aptamers selected against such targets. For that, several selection rounds against a complex target were performed. Then, a specific protein was isolated form the target and selection continued with this target. Sequences of RNA bound to this single protein were isolated and amplified. Then an affinity evaluation of such specific aptamers was performed.
Single microbead SELEX (Tok and Fischer, 2008)	According to the authors this method enables selection of high affinity-aptamers only within a few rounds of selection. A single microbead with an immobilized protein target is incubated with the oligonucleotide library. Then bound sequences are subjected to PCR amplification with fluorescently labeled primers. The PCR products are captured on streptavidin-coated microbeads and fluorescently labeled DNA is eluted. The binding of fluorescently labeled DNA with a target-coated bead is then monitored by a fluorescence stereomicroscope that also enables determination the K_d for each enriched selection cycle.
Sol–gel SELEX (Park et al., 2009)	The method uses a specially fabricated advanced device utilizing microfluidics for aptamer selection. Moreover, for protein immobilization, there are sol–gel arrays with droplets on the tops of individual microheaters of such microfluidics device used. After the target is immobilized, the solution containing oligonucleotide library is injected into the device and incubated with the target. Then each agarose droplet is individually heated and RNA aptamers are separately eluted, reverse transcribed to cDNA and amplified by PCR. Thus, such an approach is useful when selection of multiple aptamers at one time is required.
Tissue slide-based SELEX (Li et al., 2009)	This approach enables selection of aptamers against clinical specimens. DNA oligonucleotides are incubated with slices of cancerous tissue. After incubation, the complex of DNA with the cancer tissue is scraped from the slide and the oligonucleotide sequences are used in PCR amplification. In the second round of selection, the PCR products are incubated with normal tissue slices as a counter selection step. The unbound oligonucleotides are again incubated with the cancer tissue. Cloning and evaluation of affinity steps follow after 12 rounds of selection.
FACS-SELEX (Mayer et al., 2010)	A simplified scheme of FACS-SELEX includes incubation of fluorescently labeled oligonucleotide library with a mixture of different kinds of cells (*e.g.* vital and dead cells). Cells can be separated by flow cytometer to dead and live cells by employing the signal for calcein AM used for staining of the cells at the beginning of the experiment. The aptamers bound only to the cells of interest are then eluted, purified and amplified. In case of live and dead cells, the aptamers associated with a vital cell phenotype are selected. Although this method is useful for selection of aptamers against suspension or primary cells (in contrast to the cell-SELEX), it is demanding for the instrumental and workflow set-up.
Multiplexed massively parallel SELEX (Jolma et al., 2010)	This method is a modified SELEX-SAGE (Roulet et al., 2002) developed for parallel processing of samples. In this newer modification, a DNA library of special barcodes (5 nucleotides in length) that uniquely identify each particular sequence of the initial library is created. The oligonucleotide library is incubated with proteins immobilized in 96-well plate. Washing, elution and PCR amplification of bound sequences follows. After five rounds of selection, the amplified DNAs are sequenced using massively parallel single-molecule sequencing. This type of sequencing allows a large number of oligonucleotides with different barcodes to be analyzed in parallel. Also, it eliminates all cloning steps common in SELEX procedures, thus, increasing the throughput of the selection.
Domain targeted SELEX (Waybrant et al., 2012)	This technique was developed for the fractalkine protein bearing a chemokine domain (domain of interest) and a mucin-like stalk that was used in a negative selection step. Thus, the target is immobilized on agarose beads and incubated with an oligonucleotide library. Then, to select aptamers against a specific domain, the target protein is heated to 95 °C. Heating eliminates the domain of interest, while the other domain of the protein remains unaffected (mucin-like stalk that lacks secondary structure). The denatured protein is exposed to the oligonucleotide library. Sequences that are specific to the target domain remain in the supernatant while non-specific binders interacting with the protein are removed. The process includes 12 rounds of selection and amplification of the enriched sequences. Although this method enabled selection of aptamers with a K_d in a nanomolar range, its use is limited by characteristics of a particular protein (with a presence of unfolded domain) and, thus, it cannot be applied to a vast majority of proteins.
Immobilization free SELEX or GO-SELEX (Park et al., 2012)	This method is based on non-specific adsorption of ssDNA by graphene oxide (GO). First the DNA library is pre-incubated with the target. Then this mixture is added to GO, unbound DNA is adsorbed by GO due to π–π stacking, while bound DNA remains in the solution. Then, DNA is separated from the target and amplified by PCR. After several selection rounds, the aptamers are forwarded to the counter selection round. Here, closely related proteins are used as a target. When added to GO, specific sequences are absorbed and sequences bound to the counter target are removed. Aptamers are then eluted from GO by addition of the target followed by separation from the target, cloning and sequencing. The method is an easy, cost-effective and rapid approach of selection of high-affinity aptamers without the need of target immobilization.
MAI-SELEX (Gong et al., 2012)	Method is intended to generate aptamers that specifically bind to different subunits of a protein. It involves two stages of aptamer selection: an affinity module and specificity module. In the affinity module, an oligonucleotide library is incubated with a target protein immobilized on magnetic beads. This is followed by magnetic separation of the beads (5 rounds). In the specificity module the aptamer pool from the first stage is incubated with the target, in which only one of the protein subunits is presented. Bound aptamers are eluted, the unbound ones are again incubated with the protein of interest and thus aptamers specific for another protein subunit are selected.

(*continued on next page*)

Table 1 (*continued*)

Name of method (reference)	Principles of the method
RAPID-SELEX (Szeto et al., 2013)	This method is a combination of conventional SELEX and non-SELEX approaches. Here, a round performed by standard SELEX protocol is followed by SELEX round where the amplification step is not included. In total twelve selection rounds are performed; K_d is measured after each selection round to monitor the enrichment efficiency. Then, the enriched aptamers are forwarded to HTS. This method reduces time of aptamer selection in comparison with conventional SELEX while retaining the same affinity aptamer to the target.
AEGIS-SELEX (Sefah et al., 2014)	In this method AEGISs (artificially expanded genetic information systems) are used in aptamer selection. These systems include natural nucleotides and additionally two modified ones that possess higher binding variation. An AEGIS-SELEX library is built from one natural and two artificial nucleotides. Then, a standard protocol of whole cell-SELEX is applied. After the twelfth selection round, aptamers are sequenced and their affinity is evaluated. Such aptamers have K_d in a nanomolar range and are intended to have higher sequence diversity and a variety of folds than standard oligonucleotide aptamers making them more similar to proteins.
ES-SELEX (Lao et al., 2014)	Epitope-specific SELEX is developed for selection of aptamers targeting a specific epitope of a protein. At first, a ssDNA library is incubated with the target and an oligonucleotide/target complex is isolated by affinity chromatography. Then oligonucleotides are eluted by imidazole, amplified by PCR and subjected to next selection round. In the sixth round, a competitor molecule of a specific epitope of the target protein elutes bound sequences instead of imidazole. Thus, only aptamers with affinity to this epitope are selected, while other aptamers remain bound to the target. After the ninth round of selection aptamers are amplified by PCR, cloned, sequenced and used for affinity evaluation.
MARAS (Lai and Hong, 2014)	MARAS stands for magnetic-assisted rapid aptamer selection. In this method, a target protein in attached to magnetic nanoparticles, incubated with an oligonucleotide library and then subjected to rotation motion produced by an external magnetic field. Then the rotational frequency is gradually increased. This enables selection of high-affinity aptamers that remain bound with the target despite shearing force created by magnetic stirring. When the frequency reaches 14 KHz, bound oligonucleotides are collected, amplified by PCR and forwarded to counter selection against streptavidin-coated beads. Then unbound sequences are collected, amplified, cloned, sequenced and used in affinity measurements. Using this method, aptamers with desired affinity can be selected.
MSD-SELEX or Particle Display selection (J. Wang et al., 2014; Zhu et al., 2014)	The workflow starts with immobilization of primer on beads through NHS-ester. Then the beads are mixed with an oligonucleotide library (one bead–one oligonucleotide sequence) in water-in-oil droplets. The following single-copy emulsion droplet PCR results in creation of monoclonal beads bearing millions of repeats of each oligonucleotide sequence. Functionalized beads are then incubated with a target. The unbound beads are removed while bound sequences are isolated, amplified and subjected to further identification and analysis. Selected aptamers are individually displayed on the bead surface, so the affinity of potential aptamers can be directly evaluated by flow cytometry that makes this method of aptamer selection rapid and easy-to-use.

and characterized by competitive ELISA, fluorescence spectroscopy and quartz crystal microbalance methods. Thus, two DNA aptamers with nanomolar Kd_s were selected by using this process (Arnold et al., 2012). Possibly, this is the simplest and the most inexpensive approach of all the SELEX methods described in this article.

8. *In silico* selection

Along with the *in vitro* aptamer generation, a rational design of aptamers has been developed rapidly. For computational docking, called *in silico* approaches of aptamer generation, numerous programs have been developed. They can be used for prediction of oligonucleotide tertiary structure, affinity, thermodynamic characteristics and target interaction models. Among such programs are Rosetta (Das et al., 2010), FR3D (Sarver et al., 2008), R3D Align (Rahrig et al., 2010), AutoDock (Detering and Varani, 2004), DOVIS (Zhang et al., 2008), Aptamotif (Hoinka et al., 2012) and MPBind (Jiang et al., 2014). Some of them have been successfully applied to aptamer 3D structure predictions of *in silico* aptamer evolution and their truncation. For example, Chushak and Stone (2009) developed a selection strategy of RNA aptamers for specific analyte binding. Firstly, a set of criteria based on preceding knowledge of the secondary structure and thermodynamic characteristics of existing aptamers were created. These assisted in preselection of the RNA library, then, the Rosetta package was used to predict aptamer 3D structure. The computational docking in DOVIS program was then performed to select aptamers with the highest binding affinity to a ligand. Docking of six aptamers with known sequences and a pool of random RNA sequences proved high reproducibility of *in silico* selection. All of the six known aptamers were obtained among the first structures by *in silico* selection, thus verifying the functionality of the technique (Chushak and Stone, 2009).

In the same year, a deep and comprehensive study of sequence-fitness landscapes for aptamers was published. It combines a microarray technology, *in silico* approach and sequence-fitness landscape modeling. This novel method for aptamer generation is called CLADE (closed loop aptameric directed evolution) (Knight et al., 2009). The principle of CLADE is based on iterative rounds of on-chip library synthesis, interaction analysis of a target protein with a DNA library and *in silico* selection and mutation of the selected sequences, which generate a new sequence set later synthesized *in vitro*. A prior knowledge of the secondary or tertiary structures can be used in the stage of initial library design. Moreover, CLADE *per se* enables prediction of the affinity of binding, because sequences of all high-affinity aptamers after each selection round are originally known, unlike during a blind selection in conventional SELEX (Knight et al., 2009). Using CLADE, highly affine aptamers against allophycocyanin (Knight et al., 2009) and thrombin (Platt et al., 2009) were generated. A genetic algorithm applied in CLADE was used for evolution of thrombin-inhibiting aptamers (Ikebukuro et al., 2006). However, only directed evolution enabled prediction of aptamer binding with a high accuracy.

An i*n silico* approach has been also used for enhancement of binding specificity of the selected aptamers to their targets. After SELEX enrichment of aptamers, their sequences are screened by a genetic algorithm, mutated *in silico* and synthesized *in vitro*. The binding affinities of the aptamers are then evaluated *in vitro* and the selected sequences undergo further *in silico* screening and mutations (Fig. 6). For example, six rounds of cell-SELEX and two rounds of *in silico* maturation were applied to obtain aptamer PmA2G02 against *Proteus mirabilis* (Savory et al., 2013). PmA2G02 results from two rounds of *in silico* maturation exhibited specificity index 36% higher than the aptamer generated by cell-SELEX. Other aptamers gained by similar approach were targeted against prostate specific antigen (Savory et al., 2010), VEGF (Nonaka et al., 2013) and *Streptococcus mutans* (Savory et al., 2014).

To conclude, an *in silico* approach helped researchers to enhance the efficiency of the selection process to make it fully standardized. The prediction of the output of the selection makes the process of aptamer generation more cost-effective. Nowadays, *in silico* selection becomes an integral part of almost all SELEX approaches.

9. Combination of SELEX with high-throughput sequencing

High-throughput sequencing (HTS) has become a relatively cheap and user-friendly method, which can be applied in common laboratory. In newly improved SELEX techniques, HTS is used after each selection round instead of classic sequencing applied after the last selection round in previous types of SELEX (Dittmar et al., 2012; Ditzler et al.,

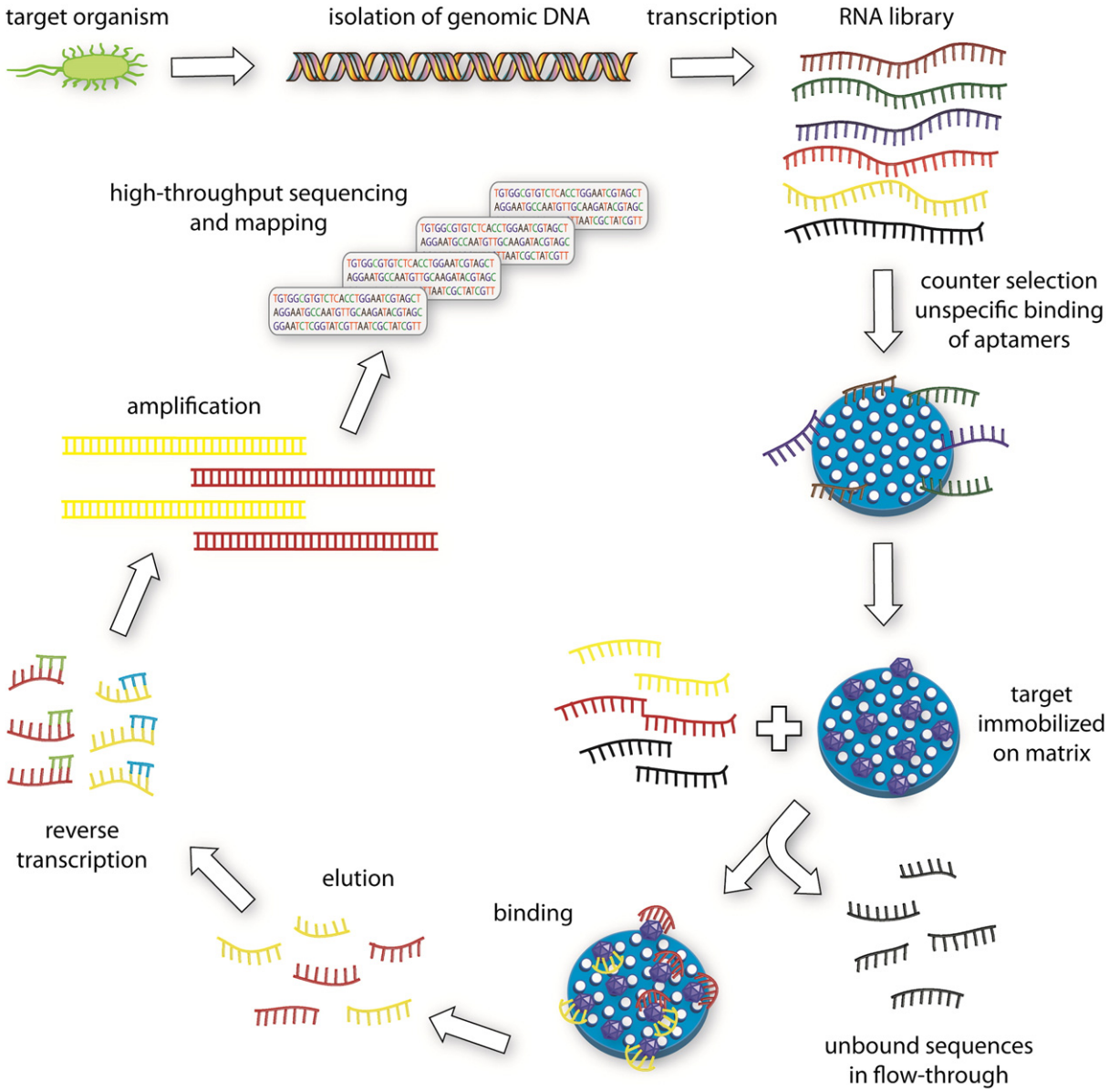

Fig. 7. Illustration of genomic SELEX. Genomic DNA is isolated from the target organism. The initial library is prepared by adding specific primers to isolated DNA and synthesis of new strands by Klenow fragment extension occurs. This initial library is then transcribed into RNA and forwarded to selection process. First, a counter selection against immobilization matrix is performed. The unbound sequences are incubated with the target. After elution, the bound oligonucleotides are reversely transcribed back into cDNA and used in further selection rounds. After several rounds of selection, enriched sequences are used for HTS and mapping analysis.

2013; Reiss et al., 2012). HTS enables comprehensive characterization of obtained aptamers, identification of their functional and rare motifs and, a comparison of functional motifs in each oligonucleotide population and quantification of their abundance. SELEX in conjunction with high-throughput sequencing, was first applied in 2002 (Roulet et al., 2002) for identification of CTF/NFI transcription factor (TF) ligands in genomic DNA. Such methods, called SELEX-SAGE, combine SELEX with a serial analysis of gene expression. In 2010, an improved SELEX-SAGE method was used to identify binding specificities for 14 different classes of TFs (Table 1) (Jolma et al., 2010).

Later, a combination of SELEX and HTS evolved into an experimental and computational platform called SELEX-seq, which can be used to determine relative affinity of a DNA sequence to TFs (Slattery et al., 2011). This approach was used to characterize binding specificity of eight *Drosophila* Hox proteins to DNA. An electrophoretic mobility shift assay was applied during three rounds of selection. HTS was performed after each selection round and computational analysis of sequencing data was applied to estimate the binding specificity of selected sequences (Slattery et al., 2011). In 2012, SELEX-seq was modified and employed for identification of binding region of ESPR1 (epithelial splicing regulatory protein 1) (Dittmar et al., 2012). A DNA library was transcribed by T7 polymerase into an RNA library, which was then incubated with a target and the bound sequences were reverse transcribed, amplified by PCR into cDNA and transcribed back into the RNA pool. The products were subjected to selection and single-end Illumina sequencing. Two to three selection rounds are sufficient to select target-binding motifs with high affinity (Dittmar et al., 2012).

HTS is also advantageous for selection of a large number of oligonucleotides. This valuable approach was employed in selection of genomic RNA aptamers recognizing various ligands. The process of enrichment of these aptamers was called genomic SELEX. It differs from the other types of SELEX by the starting pool of nucleotides. The genomic DNA library is used in genomic SELEX in contrast to chemically synthesized library in classic SELEX (Lorenz et al., 2006, 2010). Thus, the crucial advantage of genomic SELEX is a dramatic decrease of the diversity of the initial library. The genomic library is first transcribed into an RNA and

then incubated with the target protein. Secondly, the aptamers bound to the target are chromatographically separated, recovered from the target by protein denaturation, reversely transcribed into cDNA and amplified by PCR. A final pool of oligonucleotides obtained from nine selection rounds is characterized by HTS (Fig. 7). Lorenz et al. (2010) performed mapping of sequences in the *E. coli* genome and identified novel non-coding RNA aptamers binding to host factor Hfq (HF-I protein) of *E. coli*. This study has shown that genomic SELEX is a valuable technique in studying different regulatory domains of RNA.

In 2012, a method called transcriptomic SELEX bearing a striking resemblance to genomic SELEX, was published (Fujimoto et al., 2012). A total RNA pool from HeLa cells was used instead of genomic DNA in this approach. It was reversely transcribed into cDNA, amplified by PCR and transcribed back into RNA by T7 RNA polymerase. HEXIM1 protein (hexamethylene bis-acetamide inducible protein 1) binding RNA, *cad* mRNA, was identified using this approach (Fujimoto et al., 2012).

To conclude, SELEX combined with HTS has proved its extraordinary efficiency and applicability. SELEX-HTS enabled identification of different transcription factor specific binding motifs in DNA (Jolma et al., 2010) and selection of RNA sequences that bind HIV-RT (Ditzler et al., 2013). Tremendous potential for this technique remains for interaction studies.

10. Minimal primer and primer-free SELEX

Development of the genomic SELEX opened up new horizons for determination of functionally active motifs in genomic DNA. However, together with the novel approach, new obstacles have arisen. Primer binding regions in a DNA library could easily anneal with random fragments of genomic library. This is critical for the genomic SELEX since such base pairing led to a formation of loops that could be selected later as binding sites for the target, leading to inappropriate selection. Consequently, researchers created primer-free genomic SELEX, which should reduce such artifacts (Wen and Gray, 2004). The strategy of primer-free SELEX included synthesis of the genomic library, its isolation and selection of aptamers. All primers were removed from the DNA library before selection by endonucleases and the selection was performed only by using the genomic regions. After each selection round, the primer regions were re-ligated to the genomic fragments and used for PCR amplification of selected sequences. Genomic aptamers against bacteriophage fd gene 5 protein (g5p) were selected in this way (Wen and Gray, 2004).

Primer-free genomic SELEX was further modified by Pan et al. (2008). This group has developed two DNA-based methods: reducing the length of primers (minimal primer SELEX, MP SELEX) and completely eliminating primers from the target-binding step (primer-free genomic SELEX, PF). Crucial advantages of minimal primer and primer-free genomic SELEX exclude the interferences potentially caused by the primer sequences. In MP SELEX, the initial dsDNA library contained sites for two endonucleases: one site at 5′ end for *Nt.BstNBI*, a site specific endonuclease cleaving only one DNA strand on a double-stranded DNA substrate and the second site for *NotI* at the 3′ end. The final 31-nt long product generated by the nuclease cleavage consists of random DNA region flanked by 2 nucleotides at both ends. This library was incubated with skin fibroblasts as a counter-selection step. The unbound DNA mixture was then removed and incubated with melanoma cells SK-MEL-31 chosen for selection. The cell-bound DNAs were collected, hybridized with their primers by hybridization-ligation process, amplified by PCR and applied in another selection round.

Pan et al. (2008) described two similar protocols for primer-free genomic SELEX, PF1 and PF2. In PF2, only random 31-nt region generated by *BspMI* and *Nt.BstNBI* endonuclease cleavage of dsDNA library were subjected to selection, whereas, in PF1 protocol, the sequence consisted of random region flanked by 2 nts at the 3′ end. This sequence was

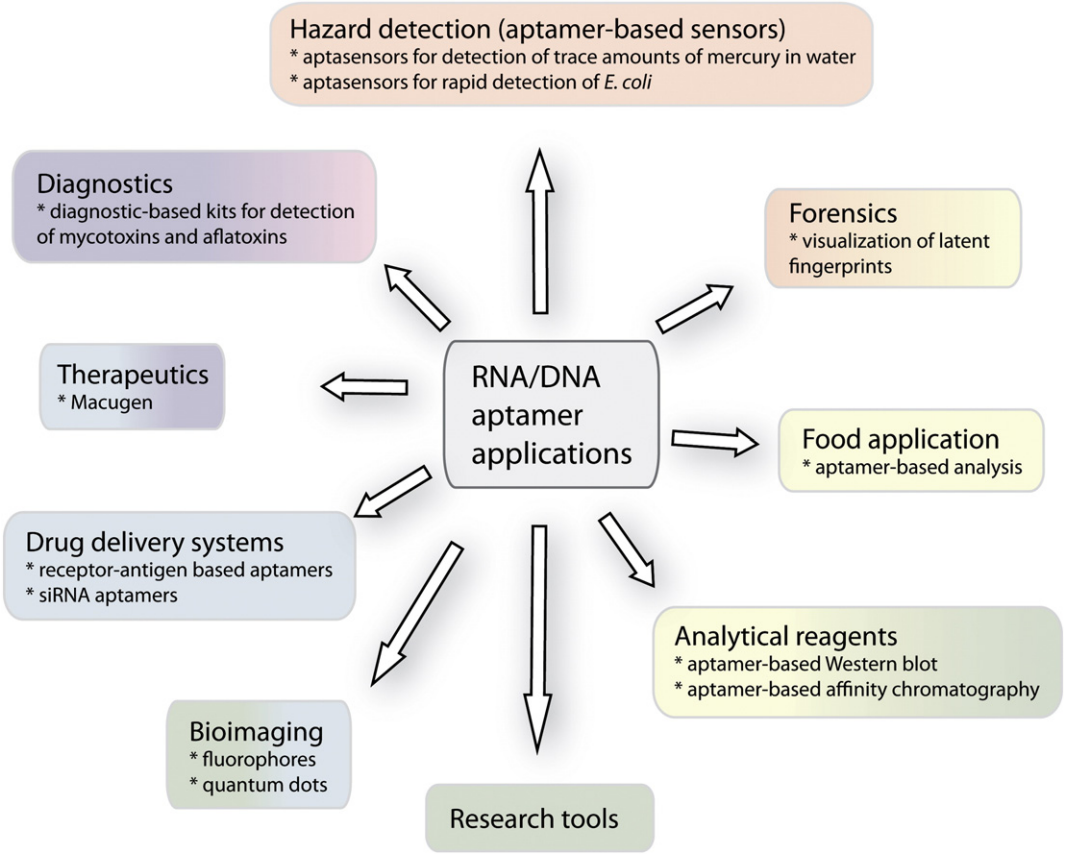

Fig. 8. Fields of aptamer applications.

obtained by cleavage with *Nt.BbvCI* and *Nt.BstNBI* endonucleases. Both libraries were incubated with melanoma cells and hybridized-ligated with the sequence containing the complement of the random region and the 5′ and 3′ flanking sequences. Selected sequences were attached to primers containing a SP6 promoter site, which was used as a starting point for the *in vitro* transcription. Then, the remaining DNA was digested with DNase I and the RNA transcript was re-amplified by RT-PCR followed by a next selection round. Aptamers obtained as the end products of all three described protocols can be used as specific surface markers of melanoma cells. By using both PF1 and PF2 protocols, Pan et al. (2010) selected an aptamer specific to human S100 calcium binding protein B (purified His6-tagged), which exhibited K_d 10^{-7}–10^{-8} M. Another variant of primer-free SELEX employing thermo-stable RNA ligase to increase the ligation efficiency of primers to random regions led to selection of high-affinity aptamers against HIV-1 RT (Lai and DeStefano, 2011).

In regard to the high demand for novel aptamers in various fields of both science and medical practice, the number of selection methods is continuously growing. In this article, we described widely used and easy to apply SELEX modifications. Other SELEX approaches, *e.g.* newly established or rarely used, are briefly described in Table 1.

11. Novel approaches to affinity evaluation during aptamer selection

Many of the previously described methods require an affinity evaluation of the isolated oligonucleotide sequences prior to choosing the aptamer with the highest affinity. This process is usually time-consuming and requires professional skills. Lately, an interesting and extraordinary method for screening of aptamer affinity after the selection has been proposed by Dausse et al. (2011). This HAPIscreen method (high-throughput aptamer identification) is based on AlphaScreen® technology by PerkinElmer; first described as a luminescence oxygen channeling immunoassay (Ullman et al., 1996). The principle of HAPIscreen method consists of a conjugation of the target molecule and potential aptamer with donor and acceptor microbeads. When the aptamer interacts with its target molecule, a fluorescence reader detects a fluorescent signal produced by microbeads. This method has been automated, thus hundreds of reactions can run simultaneously. This blind screening of aptamers allows for selection of high-affinity aptamers within a couple of hours (Dausse et al., 2011).

Recently, another remarkable method for evaluation of aptamer affinity has been published (Zhang et al., 2012). Agarose droplet microfluidic technology employed for screening of DNA library enriched during SELEX. At first, each ssDNA was packed to a single agarose droplet using flow-focusing microfluidic device. Single-molecule emulsion PCR was performed resulting in monoclonality of DNA in each agarose bead. These were stained with fluorescent dye and the target binding ability of every bead was evaluated by flow cytometry. Selectivity and dissociation constants of the clones with the highest affinity were studied (Zhang et al., 2012). Large-scale synthesis of aptamer candidates, usually required after SELEX, was successfully avoided by such screening technology. This made the evaluation of aptamer binding affinity relatively cost-effective and timesaving.

12. Post-SELEX modifications

RNA molecules are often preferred in aptamer selection particularly for diversity of their three-dimensional (3-D) structures, which RNA creates more readily than DNA. This phenomenon is caused by the presence of additional hydroxyl group at 2′ position of ribose in RNA and because of their single-stranded form predominantly occurring in nature. However, a significant drawback when working with RNA aptamers is their cleavage by ubiquitous RNases. To overcome this complication and increase their resistance to nucleases, chemically modified nucleotides are usually introduced into the structure of RNA. For example, Pegaptanib, used for treatment of AMD, is a 28-nucleotide RNA based aptamer chemically modified by fluorine and the attachment of an inverted thymidine cap at 3′ end and a polyethylene glycol linker at the 5′ end. Such modifications protect this RNA aptamer from degradation (Ruckman et al., 1998).

Commonly, modifications of 2′ position of a sugar moiety are used for this purpose, such as the following: –F, $–NH_2$ and $–OCH_3$. It was shown that these modifications significantly increase nuclease resistance of RNA aptamers (Lee and Sullenger, 1997; Pagratis et al., 1997; Rhodes et al., 2000). Special mutant of T7 RNA polymerase, Y639F, was developed for introduction of –F-, and $–NH_2$-modified nucleotides into RNA sequence during *in vitro* transcription (Padilla and Sousa, 2002). Moreover, other functional groups can be modified in RNA aptamers at the same time. However, this is feasible only by chemical synthesis after the aptamer selection. For instance, oxygen substitution for sulfur in a furanose ring of a nucleotide resulted in enhanced nuclease- and thermo-stability of modified oligonucleotides (Takahashi et al., 2009; Takahashi et al., 2012). Even though, these modifications seem promising, the studies were carried out only on short oligonucleotides, thus their application in aptamer synthesis remains questionable. The main concerns relate to decreased affinity of aptamers to their targets. Another way to modify an RNA aptamer is using locked nucleic acids (LNAs). Introduction of LNAs into aptamers during chemical synthesis increases thermo-stability of oligonucleotides (Förster et al., 2012) and significantly improves resistance to nucleases (Schmidt

Table 2
List of aptamer sequences related to particular tissues or cells.

Cell biomarker/corresponding aptamer sequence	Application (reference)
Prostate specific membrane antigen/A9 aptamer: 5′-GGGAGGACGAUGCGGACCGAAAAAGACCUGACUUCUAUACUAAGUC UACGUUCCCAGACGACUCGCCCGA-3′ A10 aptamer: 5′-GGGAGGACGAUGCGGAUCAGCCAUGUUUACGUCACUCCUUGUCAAUCCUCAUCGGC-3′	Prostate cancer therapy or diagnosis (Lupold et al., 2002)
Tyrosine-protein kinase-like 7 (PTK7)/sgc8: 5′-ATCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTTAGA-3′	Specific recognition and therapy of leukemia cells (Shangguan et al., 2008)
Nucleolin/AS1411: 5′-GGTGGTGGTGGTTGTGGTGGTGGTGG-3′	Targeting and therapy of nucleolin-expressing cancer (Ireson and Kelland, 2006)
Mucin-1 (MUC1)/S2.2: 5′-GCAGTTGATCCTTTGGATACCCTGG-3′ MA3: 5′AACCGCCCAAATCCCTAAGAGTCGGACTGCAACCTATGCTATCGTTGATGTCTGTCCAAGCAACACAGACACACTAC ACACGCACA3′	MUC1-binding aptamer advances diagnosis of eye, lung and intestine cancers (Hu et al., 2012)
CD30/C2NP: 5′-ACTGGGCGAAACAAGTCTATTGACTATGAGC-3′ NGS6.0NP: 5′-CAGCTGTCTATGAGAAAATCATCCATACGG-3′	Targeting or immunotherapy of T-cell lymphoma (Parekh et al., 2013)
CD44 and CD133/CD44 Apt1 (there are also Apt2 and Apt3): 5′-GGGAUGGAUCCAAGCUUACUGGCAUCUGGAUUUGCGCG UGCCAGAAUAAAGAGUAUAACGUGUGAAUGGGAAGCUUCGAUAGGAAUUCGG-3′ CD 133-A (also exist CD 133-A58, -A35, -A21, -A15, -B, -B19): 5′-GAGACAAGAAUAAACGCUCAACCCACCCUCCUACAUAG GGAGGAACGAGUUACUAUAGAGCUUCGACAGGAGGCUCACAAC-3′	Aptamers targeting cancer stem cells (Ababneh et al., 2012; Shigdar et al., 2013)
Immunoglobulin M Heavy Chain (IGHM) TD05: 5′-ACCGGGAGGATAGTTCGGTGGCTGTTCAGGGTCTCCTCCCGGTG-3′	Diagnosis and therapy of Burkett lymphoma (Mallikaratchy et al., 2007)

et al., 2004). Next generation aptamers, called spiegelmers, developed by Noxxon Pharma, are an alternative. These artificial oligonucleotides show high physico-chemical and biological stability, particularly due to L-ribose used instead of its conventional counterpart, D-ribose (Maasch et al., 2008). As a result of the L-nucleotide backbone, spiegelmers are resistant to practically all types of nucleases. This feature, together with the fact that they can be synthesized using standard phosphoramidite chemistry, makes them attractive agents for therapeutic applications.

Employing post-SELEX modifications often causes loss of aptamer affinity to its target. That is why the affinity of chemically synthesized aptamers should be carefully monitored upon introduction of any modification. Another way to increase the aptamer stability is incorporation of chemically modified nucleotides in the oligonucleotides of the initial library for aptamer selection. Nevertheless, this approach increases the cost of the aptamer selection process.

13. Aptamer databases

The first aptamer database was published in 2000 (Ponomarenko et al., 2000) and called SELEX_DB (http://wwwmgs.bionet.nsc.ru/mgs/systems/selex/). This database was intended to collect data from SELEX experiments and arrange all up to date selected aptamers. Although this database is still available online, it is outdated and insufficient.

The aptamer database created by the Ellington Laboratory (http://aptamer.icmb.utexas.edu/) (Lee et al., 2004) is available online and contains information about particular aptamers, authors of papers reporting these aptamers, reference to corresponding journals, name of used ligands, types of aptamers, their modifications, templates descriptions, templates sequences and aptamers sequences. At the moment of the database launching, it included only 238 aptamers. At the time of writing this article the website of this database was inaccessible.

Another aptamer database, published in 2006, is called RiboaptDB (http://mfgn.usm.edu/ebl/riboapt/) (Thodima et al., 2006). It contained 370 artificial ribozyme sequences and 3842 aptamer sequences, but at the moment the website of RiboaptDB database is non-functional.

HTPSELEX database (Jagannathan et al., 2006) (http://www.isrec.isb-sib.ch/htpselex and ftp://ftp.isrec.isb-sib.ch/pub/databases/htpselex/) was created to combine all data of the binding sites of transcription factors obtained through high-throughput SELEX experiments. Authors declared this database contained information about proteins, laboratory protocols, sequencing data and data about selected protein-binding tags. At present time, this database is unavailable online.

In 2012, an aptamer database called Aptamer Base was reported by Cruz-Toledo et al. (2012) (http://aptamer.freebase.com). This database includes information about an aptamer sequence, its secondary structure, method of generation, *etc.* Aptamers published from 1990 till 2006 were included in that database straightaway, more recent aptamers are being updated now.

Last year was published OnTheFly database containing comprehensive information about transcription factors of *Drosophila melanogaster* and its DNA-binding sites (http://bhapp.c2b2.columbia.edu/OnTheFly/index.php) (Shazman et al., 2014). This database is based on data from B1H, DNase I and SELEX experiments and provides structures or homology models of most of the included transcription factors. The authors claimed the database would be constantly updated with new information about other transcription factors whenever it becomes available.

14. Applications of aptamers selected through SELEX

Past decades of SELEX development enabled obtaining highly specific aptamers with advanced properties, such as low nanomolar Kd, multiple targeting or nuclease resistance. These newly acquired features had a great impact on application of aptamers in various fields of research, diagnostics and therapy. There are several reviews comprehensively describing the application of aptamers in these fields (Dassie and Giangrande, 2013; Kim and Gu, 2014; Meyer et al., 2013; Pei et al., 2014; Ray et al., 2013; Thakur and Ragavan; 2013; Wang and Farokhzad, 2014; Zhang et al., 2013). Fig. 8 demonstrates an overview of various fields, where aptamers are being already successfully used.

Biomedicine is one of the most important fields of aptamer application. Several aptamers were developed to recognize healthy or cancer cells. Such aptamers were mostly selected through cell-SELEX using cancer biomarkers as targets. Table 2 demonstrates some of aptamer sequences in relation to a specific organ, tissue or cells.

Many of such aptamers are intended for use in therapy of malignant diseases. They are in several stages of development from pre-clinical studies to clinical trials, and even as FDA (Food and Drug Administration) approved therapeutics. So far, it has been possible to trace about 30 clinical trials of aptamer-based drugs. Table 3 gives a description of aptamers involved in clinical trials according to https://clinicaltrials.gov/ct2/results?term=aptamer&pg=1 (14th January 2015).

Moreover, various preclinical experiments of aptamers on animal models revealed their high potential in targeted delivery of nanoparticles, therapeutics and imaging agents to various cells and molecules. The description of aptamers and conducted studies is presented in Table 4.

15. Limitations of SELEX and aptamers

When choosing a SELEX protocol to be established in a laboratory, several factors should be taken into account such as the following: availability of area designed for work with RNA (as RNA aptamers are usually preferred), available equipment for aptamer preparation, testing and purification varying according to desired SELEX modifications, preferred K_d of obtained aptamers, nature of the target, time- and cost-efficiency of the chosen SELEX protocol, need of post-SELEX modifications of selected aptamers, *etc.* So far, any ideal SELEX protocol does not exist and researchers have to choose among a variety of SELEX modifications, each of which has its own limitations and pitfalls. First of all, the cost of aptamer selection process should be taken into consideration. Thus, to start a selection process, researchers have to synthesize a massive DNA oligonucleotide library consisting of about 10^{15} sequences (James, 2000). For this purpose a laboratory has to be equipped with a robotic station for chemical synthesis. Another option is purchasing of the commercially available library; however, this greatly increases the cost of an aptamer selection. Often, RNA aptamers are preferred over DNA ones due to their ability to fold into more complex 3D structures. In this case, the initial DNA library should be converted into RNA by *in vitro* transcription, which increases not only the cost of production, but also its length. Moreover, RNA aptamers could hardly be selected by SELEX methods that utilize microarray platforms because of their high vulnerability to degradation by nucleases and shortage of RNA microarray platforms.

To minimize the selection time of aptamers, several SELEX modifications have been developed, some of them even offering automatization of the SELEX process (*e.g.* CMACS device or MMS chip described above) (Lou et al. 2009; Oh et al., 2009). Automatization might provide significant advantages in aptamer selection, enabling a large scale production of novel aptamers and decreasing the time of selection. But flexible devices that enable such automatic selection have not been produced commercially yet, thus each research group has to fabricate such devices on their own, which is a laborious and expensive process.

Another important criterion of a SELEX protocol is the affinity of selected aptamers. Several modifications of SELEX provide different affinities of generated aptamers with K_d values ranging from pM to mM concentrations (Elshafey et al. 2014; Kim et al., 2014; Lin et al. 2014). Even though SELEX modifications that provide aptamers with K_d at pM and nM concentrations are usually preferable, the SELEX protocol that generates aptamers with mM K_d could be a method of choice when the time- and cost-efficiency are limiting factors for aptamer

Table 3
Aptamers undergoing clinical trials.

Name of an aptamer (ClinicalTrials.gov identifier)	Description of clinical trials
Healthy cells	
REG1 anticoagulation system (NCT00113997 — completed)	REG1 is a regulatable aptamer-based inhibitor of factor IXa. It consists of pegnivacogin, an RNA aptamer selective factor IXa inhibitor, and of its complementary controlling agent, anivamersen. It was developed for treatment of percutaneous coronary intervention and acute coronary syndrome. REG1 is currently in Phase II clinical trials, funded by NIH.
Blood and lymph conditions	
NOX-H94 (Lexaptepid pegol) (NCT02079896)	Lexaptepid pegol is a L-stereoisomer RNA aptamer developed by Spiegelmer® technology. It binds and neutralizes hepcidin, the human peptide hormone. Hepcidin regulates iron metabolism and is a key mediator of iron restriction in anemia of chronic disease by blocking iron inside cellular stores *via* interaction with ferroportin, an iron export channel. Phase I clinical trials are currently underway.
ARC1779 anticoagulation system (NCT00632242 — completed NCT00694785 — withdrawn)	ARC1779 is a pegylated DNA aptamer that inhibits the pro-thrombotic function of vWF (von Willebrand factor) by binding to the A1 domain of vWF and, thereby, blocking its interaction with the platelet GPIb receptor. ARC1779 is expected to normalize platelet dysfunction and prevent the thrombotic end-organ complications of thrombotic thrombocytopenic purpura (TTP). Phase II clinical trials have been already completed.
NU172 anticoagulation system (NCT00808964 — unknown status)	NU172 is a DNA aptamer, which was selected to bind and inhibit thrombin, a serine protease and a key activator of several proteins in coagulation cascade. The status of Phase II clinical trials of anticoagulation in treatment of heart disease is currently unknown.
BAX499 (ARC19499) (NCT01191372 — terminated)	BAX499 is an anti-TFPI pegylated aptamer that binds to tissue factor pathway inhibitor (TFPI) and inhibits its function as a negative regulator of coagulation. Phase I of clinical trials called "First-in-Human and Proof-of-Mechanism Study of ARC19499 Administered to Hemophilia Patients" was terminated.
Cancers and other neoplasms	
Nab-Paclitaxel (Abraxane®) (NCT01830244)	Paclitaxel is one of the most potent anticancer agents used for the treatment of several cancers, including breast, ovarian, and lung cancers. Paclitaxel is a lipophilic molecule and is virtually insoluble in water. Human albumin-coated paclitaxel nanoparticles (Abraxane® or nab-paclitaxel) enable the use of toxic solvents. Nab-Paclitaxel is currently in Phase II clinical trials for newly diagnosed breast cancer. Nab — nanoparticle albumin bound
AS1411 (NCT01034410 — terminated)	AS1411 is a G-rich DNA aptamer considered to be the first anticancer aptamer. It targets nucleolin, a protein which is overexpressed in many tumor types. Phase II studies of the AS1411 combined with Cytarabine in the treatment of patients with primary refractory or relapsed acute myeloid leukemia has been terminated.
NOX-A12 (Olaptesed pegol) (NCT01194934 — completed NCT00976378 — completed)	NOX-A12 is a pegylated L-RNA aptamer developed by Spiegelmer® technology. It binds and neutralizes the human chemokine CXCL12, also named SDF-1, inhibiting signaling *via* its two receptors (CXCR4 and CXCR7). Blocking the SDF-1/CXCR4/CXCR7 axis could increase the sensitivity of tumor cells to chemotherapy and in some solid tumors could prevent tumor invasion and metastasis. Phase I clinical trials for the treatment of hematopoietic and autologous stem cells transplantation have been completed.
EYE001 (NCT00056199 — completed NCT00021736 — completed)	EYE001 is anti-VEGF pegylated aptamer developed to reduce retinal thickening and improve vision in patients with Von Hippel–Lindau syndrome (VHL). Angiomas (blood vessel tumors) commonly develop in the back of the eye on the retina and the optic nerve in patients with VHL. EYE001 decreases production of VEGF (vascular endothelial growth factor), a growth factor that is important for the formation of new blood vessels and that is elevated in VHL. Phase I clinical trials, funded by NIH, have been completed. EYE001 was also investigated for neovascular AMD treatment and Phase II/III study has been already completed.
Age-related macular degeneration (AMD)	
E10030 (Fovista®) (NCT01944839 NCT01940900 NCT01940887)	Fovista® is an anti-PDGF-β pegylated DNA aptamer, which targets platelet-derived growth factor (PDGF), a critical protein of maturation and recruitment of pericytes, for inducing a neovascular regression in AMD. Currently, Phase III clinical trials are ongoing. They are intended to establish the safety and efficacy of intravitreous administration of Fovista® administered in combination with either Lucentis® (ranibizumab) or Avastin® (bevacizumab) or Eylea®(aflibercept) compared to Lucentis® or Avastin® or Eylea® monotherapy.
ARC1905 (NCT00950638 — completed NCT00709527 — completed)	ARC1905 is the anti-complement component 5 pegylated RNA-aptamer that inhibits C5 (component 5), a central component of the complement cascade, which plays multiple roles in innate immunity and inflammatory diseases and whose aberrant activation is implicated in the pathogenesis of both exudative and dry (nonvascular) AMD. Phase I clinical trials have been completed as well as Phase I clinical trials of ARC1905 combination therapy with Lucentis®.
Diabetic macular edema (DME)	
Macugen (Pegaptanib sodium) (NCT01487044 — unknown status NCT00312351 — terminated NCT00215670 — completed NCT00321997 — completed NCT00040313 — completed NCT01487070 — completed)	Pegaptanib sodium is an anti-VEGF pegylated RNA aptamer. It is used to treat AMD (Phase IV clinical trials are almost complete), DME (clinical trials are funded by industry and other sponsors) and proliferative diabetic retinopathy (Phase 1 clinical trials have been completed). These retinopathies are accompanied by increased VEGF-based vascularization of the retina, causing blurry, myopia and blindness. Macugen was the first aptamer to enter clinical trials and obtain FDA approval in 2005 for treatment of retinopathies (Cunningham et al., 2005).
NOX-E36 (Emapticap pegol) (NCT00976729 — completed)	NOX-E36 is an L-RNA pegylated aptamer, developed by Spiegelmer® technology, that binds and neutralizes the human chemokine CCL2, also referred to as MCP-1 (C-C chemokine ligand 2/monocyte chemoattractant protein 1), and related chemokines. This aptamer was developed for the treatment of complications arising from diabetes and, specifically, diabetic nephropathy. Phase I clinical trials for the treatment of Type 2 Diabetes Mellitus, Chronic inflammatory diseases and Systematic Lupus Erythematosus have been already competed.

selection. For example, a simple one-step selection on glass coverslips might be chosen instead of SPR-SELEX due to its easier, faster and less expensive applicability, even though the aptamers chosen through SPR-SELEX would most likely have higher affinity to their targets.

Type of a target and its purity are also critical for aptamer selection. Usually, targets for SELEX are obtained by expression in prokaryotic or eukaryotic cells followed by chromatography purification. Sometimes, aptamers obtained against targets expressed in prokaryotic systems fail to bind to the same target expressed in eukaryotic cells. This may occur due to the lack post-translational modifications in the latter cells (Liu et al., 2009). On the other hand, cell-SELEX technology enables selection of aptamers without knowledge about the target. But as in the case of other methods, cell-SELEX has also its pitfalls. For example, presence of dead cells in a suspension used for aptamer generation led to nonspecific uptake or binding of oligonucleotides by these cells that had a negative impact on whole selection process (Avci-Adali et al., 2010). Microbeads were a possible solution for dead cells removal. Such removal might help to decrease the number of nonspecific aptamers obtained through cell-SELEX. In our opinion, introduction of a counter selection against dead cells might be another, maybe even more cost-effective, alternative to decrease the number of nonspecific aptamers generated by cell-SELEX.

Table 4
Examples of studies of aptamers on animal models.

Applications of aptamer	Description of the preclinical experiments on animals
Aptamers conjugated to nanoparticles (NPs)	
PSMA-targeted NPs for drug delivery	Docetaxel (Dtxl)-encapsulated nanoparticles (NPs) were formulated with biocompatible and biodegradable poly(D,L-lactic-co-glycolic acid)-block-poly(ethylene glycol) (PLGA-b-PEG) copolymer and surface functionalized with the A10 2′-fluoropyrimidine RNA aptamers. Such A10 aptamers recognize the extracellular domain of the prostate-specific membrane antigen (PSMA), a well characterized antigen expressed on the surface of prostate cancer cells (Dtxl-NP-Apt), injected intratumorally, caused tumor reduction in five of seven LNCaP xenograft nude mice and 100% of animals survived 109-day study (Farokhzad et al., 2006). After a systemic administration, A10 aptamer-targeting induced tumor accumulation of PLGA NPs by 3.7 fold when compared to the non-targeted particles (Cheng et al., 2007). The hydrophobic Pt(IV) compound 1 was used as a prodrug for delivery of cisplatin to prostate cancer using PSMA-targeted PLGA-b-PEG-NPs. The PLGA-b-PEG-Pt(IV) prodrug NPs increased the drug maximum tolerated dose when compared to that of cisplatin administered in its conventional dosage form in both rat and mouse models. A dosage of 0.3 mg/kg of aptamer-targeted cisplatin NPs was more efficacious than a 1 mg/kg dosage of free cisplatin in PSMA expressing tumors (Dhar et al., 2011).
Muc1-targeted quantum dots (QDs)	DNA aptamers developed against the highly immunogenic epitope of the un-glycosylated protein core (AptA) of the MUC1 glycoprotein and malignant glycosylated form (AptB) of MUC1 have been conjugated to MAG_2 (2-Mercaptoacetylglycylglycyl) chelator and labeled with ^{99m}Tc. This was done due to their potential use as radiopharmaceuticals for diagnostic imaging of breast cancer. These aptamers were intravenously injected to mice with MCF7 xenografts. Imaging signal accumulated in the tumors (Pieve et al., 2009). Because QD scaffolds allow labeling a single QD with multiple aptamers, targeting efficiency may be enhanced by avidity. In the female athymic mice, subcutaneously transplanted into the flanks by A2780/AD multidrug resistant human ovarian carcinoma cells, more QD-Muc1 accumulated in the tumors when compared to non-modified QD (Savla et al., 2011).
Aptamers in RNA interference	
A10-targeted siRNA	These are chimeric RNAs designed to specifically target cells expressing PSMA receptor and silencing one of the survival genes PLK1 (A10-Plk1) and BCL2 (A10-Bcl2). Upon intratumoral administration in a mouse PSMA-positive xenograft model, both aptamers specifically inhibited tumor growth and mediated tumor regression (McNamara et al., 2006). Second-generation optimized PSMA-Plk1 chimera resulted in a pronounced regression of PSMA-expressing tumors in athymic mice after systemic administration. Anti-tumor activity was further enhanced by pegylation, which increased the chimeras' circulating half-life (Dassie et al., 2009).
A10-3-targeted radiation-sensitizing shRNAs	DNAPK shRNAs, delivered by PSMA-targeting RNA aptamers, selectively reduced DNAPK (DNA-activated protein kinase, catalytic polypeptide identified as an ideal radiosensitization target) in LNCaP (human prostatic adenocarcinoma cells) xenografts established subcutaneously in nude male mice. Intratumorally administered aptamer-targeted DNAPK shRNAs, combined with ionizing radiation (IR), enhanced PSMA-positive tumor response to IR (Ni et al., 2011).
Aptamer-targeted siRNAs for treatment of HIV	Anti-gp120 (anti-HIV-1 envelope protein) aptamer or the aptamer-siRNA chimera intravenously injected to the HIV-1, infected humanized mouse model (RAG-hu), where HIV-1 replication and $CD4^+$ T cell depletion mimic the situation seen in human HIV-infected patients, suppressed HIV-1 replication and prevented the viral-induced helper $CD4^+$ T cell decline (Neff et al., 2011). CD4 aptamer-siRNA chimeras (CD4-AsiCs) specifically suppressed gene expression in the female genital tract of humanized mice. When applied intravaginally to humanized mice, CD4-AsiCs protected against HIV vaginal transmission (Wheeler et al., 2011).
Aptamers conjugated to imaging agents	
Activatable fluorescent aptamer for cancer imaging	Activatable aptamer probe (AAP) was designed to target the membrane proteins on CCRF-CEM living cancer cells and achieved contrast-enhanced cancer visualization inside mice bearing CCRF-CEM tumors. The optical signal was quenched when it was in the unbound state, but once the aptamer bound its target, a conformational change occurred with dissociation of the quencher from the fluorophore (Shi et al., 2011).
Tenascin-C targeted nuclear for cancer imaging	TTA1, an aptamer to the extracellular matrix protein tenascin-C, conjugated to MAG_2 chelator and radiolabeled with ^{99m}Tc was used to image mice bearing U251 glioblastoma tumors or MDA-MB-435 breast tumors. Moreover, different chelator modifications were tested for their effects on biodistribution and pharmacokinetics (Hicke et al., 2006).
Elastase-targeted nuclear imaging of inflammation	^{99m}Tc-labeled NX21909 (DNA aptamer inhibitor of elastase that binds preferentially to activated neutrophils) was specifically accumulating in the site of inflammation while excess NX21909 rapidly cleared from circulation in rat inflammation model (Charlton et al., 1997).

Many SELEX modifications like bead-based SELEX, microfluidic SELEX, MonoLEX, *etc.*, require immobilization of a target on a matrix. In this case, introduction of a counter selection step enables removal of nonspecific aptamers bound to immobilization matrix. Moreover, when performed with structurally similar molecules, counter selection helps to minimize an aptamer cross-reactivity (Gening et al., 2006; Lakhin et al., 2012). Such counter selection might greatly improve aptamers used as therapeutics, thus decreasing adverse effects caused by non-specific binding of aptamers to structurally related molecules. At the same time, an introduction of these additional counter selection steps might have a negative economic impact on selection of new aptamers, increasing the time and cost of overall selection process, which are a limiting factors in a large-scale development of new aptamers.

There is a number of shortcomings limiting a wider use of aptamers as therapeutics. The first of them is a rapid degradation of aptamers in blood by nucleases. This problem might be overcome by incorporation of modified nucleotides into aptamer sequence, which is described in the section on Post-SELEX modifications. Moreover, to increase aptamer lifetime in blood stream and to prevent their rapid clearance by renal filtration, usually a polyethylene molecule is added to the aptamer (Bouchard et al., 2010; Pasut and Veronese, 2012). Such conjugation increases the molecular weight of the aptamer and prolongs its lifetime in blood. All these modifications significantly increase the production cost of such aptamers.

Toxicity of aptamers and control over their action still remains an open question. So far, toxicity studies have been performed on two aptamers, Pegaptanib and AS1411 (Bouchard et al., 2010; Ireson and Kelland, 2006). Low toxicity rate was shown for both aptamers. In general, aptamer toxicity should be determined prior to its use as therapeutics. Considering the fact, that many aptamers undergo various stages of clinical trials (Sundaram et al., 2013; https://clinicaltrials.gov/ct2/results?term=aptamer&pg=1), more information about their toxicity should be available in near future. The control of aptamer action is another challenge in their therapeutic applications. Several strategies were introduced to achieve prolonged aptamer action. One of them is injection of antidotes into a bloodstream that create complexes with aptamers and inactivate them (Rusconi et al., 2002), another strategy is creation of aptamers that become active only under certain conditions, for example, after light illumination when aptamers become active by splitting off a photosensitive part (Brieke et al., 2012; Buff et al., 2010).

In conclusion, the variety of developed SELEX modifications provides an opportunity to establish aptamer selection in any laboratory. Though still expensive, SELEX becomes more cost-effective, less labor-intensive and provides higher yield of selected aptamers. Moreover, due to the

recent SELEX development, aptamers have even higher affinity to their targets than at the beginning of SELEX era. High resistance to nucleases obtained *via* post-SELEX modifications, low toxicity and immunogenicity make them attractive tools and stimulate constant development of new aptamers for various application fields.

16. Phage display *vs.* aptamer selection: on the advantages and pitfalls

How does SELEX differ from other popular methods used for biosensor development, such as phage display, and what are the bright and dark sites of these methods? We present some answers to this question below.

Phage display, reported by G. P. Smith in 1985 (Smith, 1985), still remains one of the most widely used molecular biology tools for generation of powerful peptide and protein libraries against targets of interest. Phage display has gained the largest attention for its successful application in isolation of monoclonal antibodies (Winter et al., 1994), which enabled to surmount the tremendously labor-demanding and time-consuming hybridoma-based method. Moreover, phage display antibodies production has not been limited anymore to production of human antibodies and antibodies against toxic (Winter and Milstein, 1991) and non-immunogenic targets. The principle lies in cloning of DNA library sequences into a gene encoding phage coat protein. Upon the expression of fusion coat protein modified with library sequences, desired peptide targets are displayed on the phage surface (Smith, 1985), thus allowing facile selection by a variety of affinity methods at desired stringency. The main advantage of phage display is the fact that both the expressed peptide and its coding DNA are available on and in the same phage particle, respectively (Smith, 1985). Therefore, the isolation and sequencing of DNA encoding particular displayed peptide is very simple. The most commonly utilized are filamentous bacteriophages, especially M13 phage infecting *E. coli* (Petrenko and Vodyanoy, 2003). M13 phage consists of major coat protein VIII (pVIII), minor coat protein III (pIII) and ssDNA coding four other minor coat proteins. Protein pIII, involved in bacterial infection, occurs only in three to five copies unlike pVIII which is present in ca 2700 copies. Hence, pIII is preferentially used for peptide or protein fusions. Excessive occurrence of a coat protein would result in enhanced target binding and thus low specificity (Falciani et al., 2005). Later, phages were replaced by specialized phagemids (phagemid display system), where protein expression was separated from phage propagation by using a helper phage. This has simplified the library amplification, ensured both higher genetic stability (Clackson et al., 1991) and transduction efficiency. The next step *i.e.* the enrichment of specific binders by so called (bio)panning (Parmley and Smith, 1988) is usually repeated three to five times. It lies in incubation of the phage library with a tested target that is either immobilized, in solution, on the cell surface or *in vivo*. Nonspecifically bound phages are washed away, and the positive ones are used for *E. coli* reinfection and amplification followed by DNA sequencing and binding verification. The first foreign proteins displayed on a phage surface were antibodies (McCafferty et al., 1990). Twelve years later an immense progress occurred resulting in marketed human antibody against tumor necrosis factor alpha (anti-TNF-α, adalimumab, commercial name Humira®) (Kempeni, 1999). Similarly as aptamers, the peptides and proteins generated by phage display stand for very specific and highly affine molecules raised against a wide range of desired targets. The phage display system contributed also to cancer research revealing a plethora of important biomarkers, agonist/antagonists and mapping epitopes. Recently it has been also applied in vaccine design (Gao et al., 2010) and used for host-pathogen interaction studies (Tonelli et al., 2013). Moreover, it might be employed in target recognition, its characterization and quantification.

Overall, phage display is a rapid and inexpensive method which has already proven its validity. Despite these facts, phage display is restraint by number of issues: i) the level of protein expression in *E. coli* strongly depends on the primary sequence, and thus it might be very low for some proteins (Hoogenboom et al., 1998); ii) not all peptides and proteins can be displayed — they might interfere with phage assembly and/or cause phage toxicity (Hammers and Stanley, 2013); iii) the diversity might be lost by competition of bacteriophages for a limited number of binding sites during the library amplification in bacteria (Henry and Debarbieux, 2012), which reduces the number of identified peptides or proteins specific for the examined target; iv) proteins identified by *in vitro* phage display may have different folding in target cells or organisms; v) phage display libraries consist only from about 10^8 independent *E. coli* transformed cells (given by the transduction efficiency).

In contrast, aptamers are selected from synthetic libraries consisting of more than 10^{15} sequences, thus assuring significantly higher heterogeneity and chance to select a specific binder compared to a less diverse phage display system. Another characteristics of aptamer selection is the fact that they are mostly generated *in vitro* which is advantageous, since the SELEX conditions (temperature, pH, ionic strength, *etc.*) might be adjusted as demanded to obtain aptamers to meet particular goals (Wang et al., 2011). The drawback is that some of the *in vitro* generated aptamers may not be functional when applied *in vivo*, similarly as in the case of phage display. Some *in vivo* SELEX applications have been already described, see Section 6 for details. Phage display was also successfully performed *in vivo*, first reported by Pasqualini and Ruoslahti in 1996. Random phage display library was injected directly into a living animal that was then sacrificed and a lysate of desired tissue was used for phage amplification (Pasqualini and Ruoslahti, 1996). A size of an *in vivo* phage display library can reach up to 10^9 of different clones (Bábíčková et al., 2013). However, it must be taken into account that survival of the phages in the experimental animal might be a key issue in terms of their functionality, because of the reticuloendothelial clearance system (RES). Therefore the phage selection is usually performed only for a very short time; details on phage biodistribution and clearance in three different mouse models (CF-1, nude, and SCID mice) can be found in Zou et al. (2004). Similar obstacles were observed for RNA aptamers by Cheng et al. (2013), which are with their size, usually ranging from 5 to 15 kDa, ideal for RES elimination. *In vivo* studies employing human phage display were performed already in 2001. After phage library injection into a patient in coma, biopsy samples were collected and analyzed (Arap and Pasqualini, 2001). Ligand of interleukin-11 receptor was one of the peptides identified (Zurita et al., 2004).

In comparison to peptides and proteins derived from phage display, wide aptamer applications are limited by their short half-life, which relates especially to RNA aptamers degraded by ubiquitous RNases (not to DNA and peptide aptamers). Nevertheless, nowadays, these obstacles can be overcome by introducing modified bases (Di Giusto et al., 2005) or using spiegelmers (Eulberg and Klussmann, 2003).

Phage display and aptamer selection are both very potent, rapid and cost-effective techniques yielding a large diversity of binders screened against possibly any target with no batch to batch variations (Mehta et al., 2011). In these cyclic repetitive methods, some binders with high affinity might be lost during amplification under improperly chosen conditions. Because of the lower cost of the DNA/RNA selected sequences and faster and more facile preparation when compared to peptide synthesis, SELEX is cheaper and more convenient. On top of that, aptamers are advantageous also for their possible reusability after a denaturation and renaturation cycle, which makes the overall process even cheaper (Mehta et al., 2011).

Besides the foremost displays employing filamentous phages, there are also other display techniques utilizing *e.g.* bacteriophage λ (Beghetto and Gargano, 2011; Gupta et al., 2003; Pavoni et al., 2013), baculovirus (Iida et al., 2013; Mottershead et al., 1997), bacteria (Benhar, 2001; Kronqvist et al., 2008; Rutherford and Mourez, 2006; Samuelson et al., 2002; Stahl and Uhlén, 1997) or ribosomes (He and Taussig, 2002; Schaffitzel et al., 1999; Zahnd et al., 2007). Ribosome,

or cell-free, display surmounts the diversity limitations caused by low transduction efficiency.

Recently, a very interesting technique combining approaches of both SELEX and phage display was reported by J. Wang et al. (2014), Q. Wang et al. (2014), and Y. Wang et al. (2014). This method called 'particle display' consists in transformation of individual aptamers into aptamer particles (using emulsion PCR), then the particular nucleic acids are displayed on their surface in large number of copies. Relative affinities of each particle are measured by FACS (fluorescence-activated cell sorting) and can be also sorted (J. Wang et al., 2014; Q. Wang et al., 2014; Y. Wang et al., 2014). Sorting of phages by FACS has not been possible because of their small size.

Such innovative methods and rapid identification of disease biomarkers at its early stage are inevitable for diagnostic interventions and appropriate treatment. In summary, both phage display and aptamers have helped to decipher fundamental biological processes, and on top the cost reduction significantly speeded up and facilitated novel antibodies and sensor production and biomarker detection.

17. Conclusions and future perspectives

A giant leap in improvement of SELEX technology has been made since its discovery in 1990. Numerous clinical applications of aptamers evolved by SELEX approaches have been described so far. These high-affinity oligonucleotides specifically binding to their targets often mimicking drugs and antibodies are promising prognostic, diagnostic and therapeutic tools. One of the classic examples of a successfully selected and applied aptamer is anti-angiogenic drug, Macugen marketed by Pfizer.

Tremendous progress in development of SELEX methods has enabled a move from synthetic chemical libraries over whole cell and tissue lysates up to *in vivo* tumors for evolution of made-to-measure aptamers. Many of those have a great potential to serve as novel biomarkers. The optimization and automation of SELEX technology facilitated and shortened whole selection procedure. Moreover, rapid progress in related fields of microfluidics, nanotechnology, novel sequencing methods and biomedicine accelerated development of to a number of novel revolutionizing SELEX techniques covered by this review.

Significant refinements are still needed to increase the biostability and bioavailability of aptamers, especially of those intended for clinical practice. Further modifications of SELEX procedure offer an incredible challenge for scientist to evolve powerful alternatives of the present-day aptamers.

Conflict of interest

No conflict of interest declared.

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