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Trends in Aptamer Selection Methods and Applications

Meral Yüce[*1] Naimat Ullah[2] Hikmet Budak[1, 2]

[1] Sabanci University, Nanotechnology Research and Application Centre, 34956, Istanbul, Turkey

[2] Sabanci University, Faculty of Engineering and Natural Sciences, 34956, Istanbul, Turkey

Abstract

Aptamers are target specific ssDNA, RNA or peptide sequences generated by an *in vitro* selection and amplification method called SELEX (Systematic Evolution of Ligands by EXponential Enrichment), which involves repetitive cycles of binding, recovery and amplification steps. Aptamers have the ability to bind with a variety of targets such as drugs, proteins, heavy metals, pathogens with high specificity and selectivity. Aptamers are similar to monoclonal antibodies regarding their binding affinities, but they provide a number of advantages to the existing antibody-based detection methods that make the aptamers promising diagnostic and therapeutic tools for the future biomedical and analytical applications. The aim of this review article is to provide an overview of the recent advancements in aptamer screening methods along with a concise description of the major application areas of aptamers including the biomarker discovery, diagnostics, imaging and the nanotechnology.

Keywords: Aptamers, SELEX, Biomarker Discovery, Diagnostics, Targeted Delivery, Nanomaterials, Imaging, Biosensors

1. Introduction

Aptamers are short, target specific ssDNA, RNA or peptide sequences generated by an *in vitro* selection and amplification technique called Systematic Evolution of Ligands by EXponential Enrichment, SELEX, using a random single-stranded DNA or RNA pool.[1,2] Since the discovery of the method, a diverse collection of aptamers for hundreds of various targets has been developed from the random pools using either traditional or the modified SELEX techniques. Aptamers have been used to probe drugs,[3] proteins,[4] heavy metals,[5] infectious bacteria[6] and even nanomaterials[7] with high affinity and selectivity.

In most cases, aptamer selection starts with a pool of RNA or DNA sequences that are completely random except for the primer binding regions. The conventional screening method, SELEX, consists of the repetitive cycles of incubation, elution and the amplification steps, as illustrated in **Figure 1**. The incubation of a given target with the random library is followed by the recovery of binding sequences that are eventually amplified and used for the next round of selection, after the single strand separation step if necessary. The art of enrichment lies in the partitioning of the target specific sequences away from the overwhelming majority of the non-specific sequences. The whole process is repeated for various rounds until the random library is enriched with the sequences of high affinity for the target, which is indeed when the start pool becomes dominated by the enriched sequences. The pool enrichment step is pursued by the sequencing step (either conventional Sanger Sequencing or Next-generation Sequencing) and the chemical synthesis of the selected aptamer candidates. Equilibrium dissociation constants (K_D) of the generated aptamer-target complexes range from low picomolar to micromolar levels that are usually defined by the techniques such as Surface Plasmon Resonance, Quartz Crystal Microbalance, pull down assays or Microscale Thermophoresis. Affinity range of aptamers depends on the capability of their three-dimensional conformation upon binding to the target molecule, as well as the target molecule itself. The global market of aptamers is expected to reach approximately $2.1 billion by 2018 according to the recent research by Markets and Markets.[8] The sustainability of growth in aptamer market depends on the technological developments with respect to the selection processes and synthesis.

This review covers the latest advancements in SELEX methods and the implementations of aptamers in several major fields such as biomarker discovery and diagnostics, targeted delivery and imaging,

food safety and environmental monitoring. The report also provides information about the aptamers in general for those who are not familiar with the field.

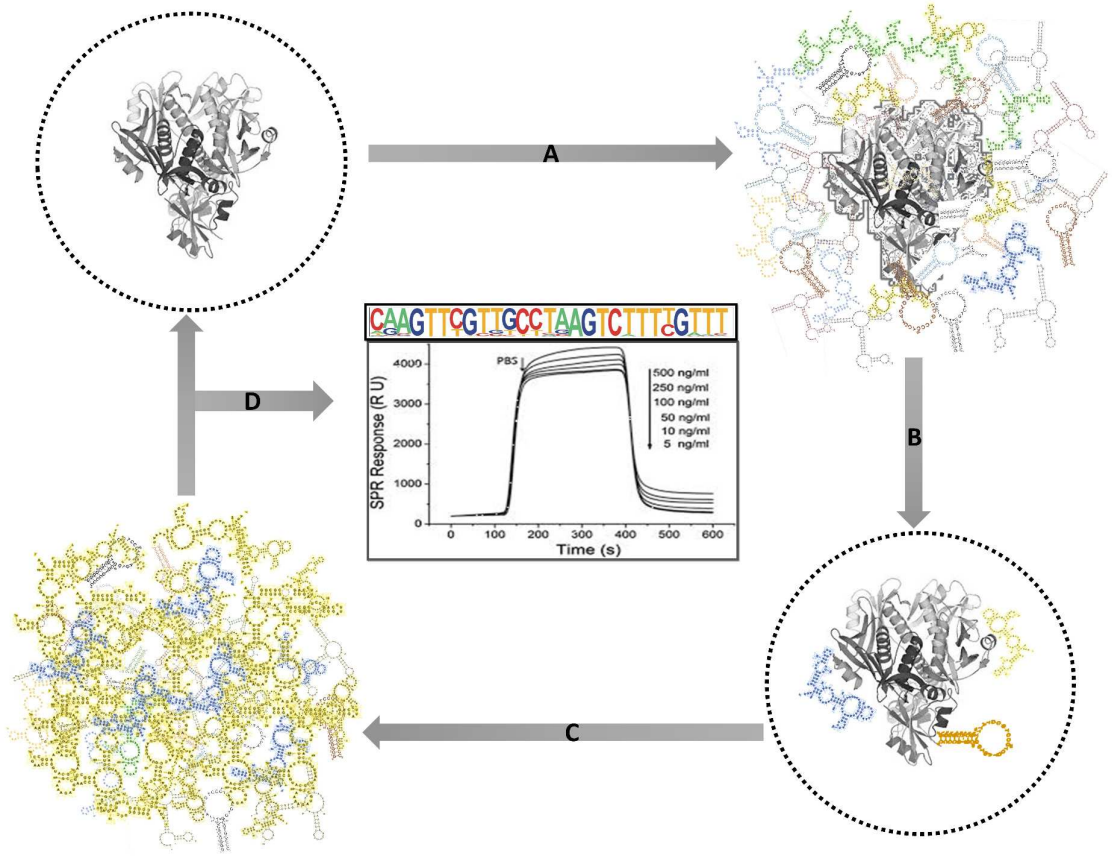

Figure 1. Illustration of a standard SELEX round. A) Incubation of the target molecule with the random oligonucleotide library, B) Elution of the target bound oligonucleotides and elimination of the non-specific binders, C) Amplification of the target bound oligonucleotides in which the tightly binding oligonucleotides dominate the pool, known as enrichment, D) Following X-cycles of the SELEX (A, B, C), the enriched pool is sequenced and analysed for the consensus sequences whose binding abilities are tested through the well-known analytic methods like SPR.

2. Exclusive Features of Aptamers

Antibodies have been used as the key probes of the proteins in various analytical formats such as western blot, microarray, ELISA, immune-bead assay since 1970s.[9] Aptamers are similar to monoclonal antibodies regarding their binding affinities but they provide faster and more cost-

effective production methods as well as the extended applications. Because of the fully controlled synthesis, target versatility, selective binding ability, thermal and chemical stability, aptamers have become the core components of the research in diverse fields from biotechnology to materials science and nanotechnology. Some key properties of aptamers are specified below:

2.1. Enabling *in vitro* production

Aptamers can be chemically generated in a test tube on demand without the conventional problems associated with the production of recombinant antibodies that are prone to viral or bacterial contamination. Aptamers can be used under a broad range of experimental conditions as a result of their thermal and chemical stability, whereas proteins are very sensitive to the environmental conditions, and they rapidly lose their tertiary structure at elevated temperatures. Moreover, aptamers can be readily synthesized in large quantities with relatively smaller cost whereas the antibody production is laborious, besides, it requires animal sacrifice.

2.2. Minor batch to batch variations

Antibodies often suffer from the batch to batch variations while aptamers are chemically synthesized without or with negligible batch variation. In addition, chemical synthesis process allows the introduction of a variety of functional groups (i.e. fluorophores) into the desired locations of the aptamers without disturbing their functions, whereas *in vitro* protein-labelling techniques can compromise both the structure and the function.

2.3. Broad target selection and selectivity

The affinity of antibodies is limited to the molecules that produce an immuno-response while aptamers can bind with hundreds of molecules at different size and structures. Successful aptamer selections have been performed on a diverse array of small and big targets including nucleotides, nucleobases, ions, cofactors, antibiotics, enzymes, organelles, proteins, tissue cells and whole microbiological samples. While antibodies have cross-reactivity and/or false positive signal issues,[10] for example, the RNA aptamer generated against the theophylline exhibits 10,000-fold greater binding affinity for the theophylline over the caffeine, which deviates from the theophylline by a single methyl group.[11] Theophylline aptamer is the first example of an RNA sequence capable of

discriminating between the small ligands with a specificity rivalling that of antibodies. Furthermore, a new class of aptamers with slow off-rates, so-called SOMAmers,[12] has been generated with sub-nanomolar affinities towards their target proteins, which are compatible with many biological samples such as human serum, plasma, cerebrospinal fluid, tissue homogenates, synovial fluid, cell lysates.[13]

2.4. Small size

The small size of the aptamers allows their penetration through the cells or biological compartments, which has been utilized in drug delivery[14] and molecular imaging[15] applications. The size of the aptamers could enable their access to more epitopes or the epitopes that are unavailable to the antibodies due to the steric hindrance. For example, the brain capillary endothelial-specific aptamers identified by Cheng et al.[16] penetrated successfully into the parenchyma cells, which was suggested to be through the adsorptive-mediated transcytosis or the fluid-phase pinocytosis. Besides, Schauen et al.[17] showed that EGFR aptamer was capable of recognizing twice as many epitopes compared with its antibody while the prostate-specific membrane antigen (PSMA) aptamer identified hundred times the number of epitopes compared with the PSMA antibody. Although the small aptamers are susceptible to the renal filtration and the enzyme degradation, they can be conjugated with high molecular weight polymers (e.g. polyethylene glycol) or nanostructures (e.g. gold and silver nanoparticles) to promote persistence in the circulation by increasing half-lives.

2.5. Facile modification and labeling

The affinity of the antibodies compromises after labelling whereas aptamers can be labelled without disturbing their affinity. Fluorescent dyes or functional groups can be readily introduced into the random libraries before, during or after the selection, which broadens the applications of aptamers. Modification of the aptamers with biotin, fluorescent probes, and thiol tags can be achieved via *in vitro* transcription or PCR methods on demand. Incorporation of the unnatural bases into the libraries to increase the final diversity and the affinity of the sequences is also possible. For example, Imaizumi and coworkers[18] introduced (E)-5-(2-(N-(2-(N(6)-adeninyl)ethyl))carbamylvinyl)-uracil bases at the C5-position of the uracil base, expecting improved chemical and structural diversity of the pool, so they can better accommodate the discreet epitopes of the target molecule. Incorporation of an unnatural nucleotide with the hydrophobic base 7-(2-thienyl) imidazole [4, 5-b] pyridine into the

random library delivered a VEGF-165 specific aptamer with a K_D of 0.65 pM.[19] Integration of 2' modified pyrimidine nucleotides into the ribose moiety, on the other hand, increased the stability of the transcribed RNA molecules since the most ribonucleases polarize the 2-hydroxyl group to attack the phosphodiester linkage. 2' chemical modification mainly covers 2'-fluoro- or 2'-amino-substituted pyrimidines or 2'-O-methyl nucleotides that enhance the nuclease resistance of the resulting aptamers.[20] He et al.[21] introduced drug-like moieties into the random libraries to increase the final stability, as well as the affinity of the aptamer candidates. The authors achieved to rise the final affinity of the CD44-HABD aptamers by 23 fold by the incorporation of N-acetyl-2, 3-dehydro-2-deoxyneuraminic acid into the 5' positions of the certain uridines in the root library while increasing the nuclease stability using a full monothiophosphate (permonothioated) backbone. These studies confirm that the incorporation of unnatural bases into the random pools can return aptamers with enhanced affinities, stabilities, and structural diversities, providing new insights into the development of next generation clinical aptamers. Further aspects of the employment of peptide/drugs like moieties or unnatural bases in the random libraries were discussed in detail by Knudsen et al.[22] and Rohloff et al.,[23] respectively.

3. Classical Techniques for Aptamer Selection

3.1. Magnetic beads

Magnetic bead-based SELEX is one of the core SELEX methods used, mainly because of the aptamer candidates bound to the target can be easily and quickly separated from the remaining reaction mixture using a magnet. Magnetic beads allow the covalent or the non-covalent target immobilization on the surface. For example, carboxylic acid functionalized magnetic beads can be modified with a target molecule that contains primary amine groups, following the surface activation step by carbodiimides (NHS-EDC) that forms a covalent bond between the target molecule and the beads. The remaining activated carboxylic groups are blocked using the reagents with free amine groups like Bovine serum albumin or Ethanolamine. Although the immobilization through the functionalized beads is straightforward, the uniform distribution of the targets on the bead surface is not always possible, resulting in conformational changes in the immobilized molecule, which ultimately affects the enrichment of the random library. Alternatively, the magnetic beads with exclusive surface properties, for example, Ni-NTA, Co-NTA or Streptavidin coated beads, can provide

higher binding capacities with very little background noise for proteins with special tags. The specific surface chemistries also enable proteins to be aligned with a particular orientation on the surface. However, in either case, the potential free sites on the bead surface left after immobilization, are still prone to bind oligonucleotides, which paves the way for the non-specific enrichment of the random library. Thus, a negative selection step against the separation/immobilization surface is always recommended between the rounds in order to eliminate the oligonucleotides that are unique to the surface rather than the target molecule itself. It should be noted that there are also aptamers generated against particular immobilization surfaces, i.e. histidine, ethanolamine[24] or streptavidin[25] recognizing aptamers, which could be employed to reduce the surface-specific oligonucleotide enrichment during selection. Magnetic beads are not used only to immobilize the SELEX targets[26] but also to capture[27] or generate the single-stranded DNA library[28] during the selections. An interesting application of the magnetic beads in SELEX was the utilization of a single magnetic bead functionalized with the target molecule.[29] The authors applied only two rounds of selection, and they simultaneously controlled the binding of the fluorescently labelled ssDNA pool to the target-functionalized bead under the fluorescence stereo-microscope, where the aptamers selected against the *Clostridium botulinum* neurotoxin-related targets (Hc-peptide and the Toxoid) showed nanomolar affinities.

3.2. Capillary electrophoresis

Capillary electrophoresis-based SELEX is a quick selection method that enables the selection of aptamers within 2–4 rounds, unlike many other screening methods. It has some unique characteristics over the other analytical separation methods including the high speed, resolution, capacity and minimal sample dilution. Capillary electrophoresis can separate even the tiny molecules like porphyrin[30] under an electric field, based on their few properties like the charge and hydrodynamic radius. Unlike other SELEX methods, the random library is freely incubated with the target molecule in the binding buffer, facilitating the capture of the targets in their native three-dimensional structures, without using labels, separating columns or resins. The incubation media is later loaded into a capillary electrophoresis system in which the target bound aptamer candidates, and the free oligonucleotides migrate at a distance, allowing their collection as different fractions. The following steps are the purification and the amplification of the bound oligonucleotides for the next round, which are the same steps as in the conventional SELEX. However, the injection volume

limit in capillary systems (usually 5-50 ul) constrains the use of libraries at the maximum sequence diversity since they severely exceed the capillary threshold, complicating the separation of the bound and the unbound molecules. In conventional SELEX, random libraries containing 10^{13-15} sequences can be used for the screening, because, the maximum diversity of the starting pool could yield better aptamers. Nevertheless, the number of different sequences is expected to decrease after the very first round, which improves the quality of the resolution at subsequent rounds of the capillary electrophoresis-based SELEX.

3.3. Whole cell-SELEX

Whole cell-SELEX exploits an entire cell as the target, whereas the primary targets for the other SELEX methods are highly-purified targets. Extracellular cell surface proteins or some unknown structures of the cells can be the targets of the cell-SELEX process. Whole cell targeting aptamers can be generated through this SELEX method without having much knowledge about the surface proteins of the cells, which enables the discovery of novel biomarkers for primarily diagnostic and imaging purposes, as discussed in the following sections. Because the immobilization of the targets is not feasible in the cell-SELEX method, heating and centrifugation methods are usually used for the isolation of the oligonucleotides bound to the target cells. The technique has been used to generate a number of aptamers for especially foodborne pathogens from which the most recent ones are summarized in **Table 1**. On the other hand, specific cell surface proteins in their native forms can still be targeted using the cell-SELEX method, as recently accomplished by Meyer et al.[31] who named the method as STACS (Specific TArget Cell-SELEX). In case of the specific protein targeting with cell SELEX process, the cells are adapted to express the selected target proteins in excess to be used in capturing step, and the control cells lacking specific proteins on the surface are used for the negative-SELEX step.

Table 1: Aptamers generated against some foodborne pathogens using the whole cell-SELEX technique

Target name	K_D	5'-aptamer sequence-3'	Ref
Alive Salmonella typhimurium (Counter targets: Salmonella Enteritidis, Escherichia coli Staphylococcus aureus)	10^7CFU /3nmol aptamer	**Aptamer C4** ACGGGCGTGGGGGCAATGCCTGCTTGTAGG CTTCCCCTGTGCGCG	32
Listeria spp. *(Counter targets: E. coli O157: H7, Bacillus cereus, Salmonella enteritidis, Staphylococcus aureus, Pseudomonas aeruginosa, Shigella flexneri, Brochothrix thermosphacta, Lactococcus lactis*	74.4±52.69 nM	**Aptamer LM6-116 (targeting the cells at stationary phase)** TACTCGTTATTTCGTAGCACTTTTCCCCACCAC CTTGGTG	33
	106.4±43.91 nM	**Aptamer LM12-6 (targeting the cells at log phase)** TCTGTGTTCCGTTTTCGATTCTTACTGTGTTTT CGGGTGC	
Campylobacter jejuni strains (Counter targets: Bacillus cereus Strain T, E. coli O157:H7, Listeria monocytogenes)	292.8±53.1 nM	**Aptamer ONS-23** CAAGGGACAGTAGACCAACAGGAAATCAAA GGCGTGGGAA	34
Staphylococcus aureus (Counter targets: Salmonella typhimurium, Salmonella enteritidis, Escherichia coli)	3.49 ± 1.43 nM	**Aptamer A14** CACACCGCAGCAGTGGGAACGTTTCAGCCAT GCAAGCATCACGCCCGT	35
Escherichia coli strains (Counter targets: Klebsiella pneumoniae, Citrobacter freundii, Enterobacter aerogenes, Staphylococcus epidermidis)	12.4 nM	**Aptamer E1** ACTTAGGTCGAGGTTAGTTTGTCTTGCTGGC GCATCCACTGAGCG	36
	25.2 nM	**Aptamer E2** CCATGAGTGTTGTGAAATGTTGGGACACTAG GTGGCATAGAGCCG	
	14.2 nM	**Aptamer E10** GTTGCACTGTGCGGCCGAGCTGCCCCCTGGT TTGTGAATACCCTGGG	
	16.8 nM	**Aptamer E12** GCGAGGGCCAACGGTGGTTACGTCGCTACG GCGCTACTGGTTGAT	

3.4. Surface plasmon resonance (SPR) or flow cell SELEX

An SPR sensor comprises an optical system to excite plasmon, a transducing element to transfer the biochemical signals into the optical signals, and an electronic system supporting the data acquisition and the processing.[37] Binding of analytes onto the ligand immobilized on a SPR chip (usually gold) leads an increase in the mass causing a proportional increase in refractive index, which is eventually detected as a shift in the resonance angle. SPR technique has been used to evaluate label-free molecular interactions between the protein-protein,[38] protein-DNA[39] and protein-RNA.[40] Because of the high sensitivity and the opportunity to trace the reactions as they occur, SPR technique has become the core platform for several aptamer-based biosensors, as briefly listed in **Table 2**.

Apart from the aptamer-based sensor devices, SPR technique has been solely used as a base platform for the generation of aptamers on the chip surface, enabling the real-time detection of the binding kinetics at every single selection cycle. The technique was first described in 1999 by Schneider et al.[41] where it was called as the Flow cell-SELEX. Later, it has been applied by Khati et al.[42] to screen RNA aptamers binding gp120 protein of the HIV-1 R5 strain Ba-L (HIV-) and neutralizing the infectious virus, by Misono et al.[43] to screen aptamers for hemagglutinin (HA) of human influenza virus, and recently by Ngubane et al.[44] for the generation of RNA aptamers targeting *Mycobacterium tuberculosis* EsxG protein.

SPR-based aptamer screening technique is based on a metal chip modified with the target of interest, which is exposed to the random oligonucleotide library for the binding of the sequences. Although there are functionalized chips commercially available (e.g. streptavidin coated chips for the biotinylated targets, Ni-NTA chips for the proteins with his-tags, carboxymethylated dextran surfaces for amine coupling), a simple clean gold chip will also work well for the target immobilization. A number of methods to prepare the gold surface for target immobilization is readily available in the literature.[45] Because the SPR devices are usually equipped with a controllable microfluidic channel, the elimination of the non-specific binders can be simply achieved through the consecutive injections of the binding buffer or any other solution that would increase the stringency of the selection. Besides, the flow rate of the channels could be tuned as required, which might allow to select aptamers with slow off-rates. The elution of the target bound oligonucleotides from the chip surface is usually achieved by small volume injection (30-100 ul) of a strong reagent (e.g. NaOH, urea, EDTA, Glycine HCl) that could break the 3D structure of oligonucleotides, enabling their collection from the

output microvalves as they leave the surface. Centrifugal filters or charge switch magnetic microspheres would be practical options to desalt or concentrate the elution at this step while the traditional methods like ethanol precipitation are also effective. Following the purification/precipitation of the oligonucleotides from the collected fractions, amplification and the ssDNA production steps (or *in vitro* transcription step, in the case of RNA aptamers) are applied as in the conventional SELEX methods.

Table 2: Examples of some SPR-based aptamer biosensors and the associated aptamer sequences

Analytes	LOD	Ref	5'-Aptamer sequence-3' used	Ref
Cytochrome c detection for anti-cancer drug screening	≥50 pM	[46]	**Aptamer Clone 3** ATCGATAAGCTTCCAGAGCCGTGTCTGGGGCC GACCGGCGCATTGGGTACGTTGTTGCCGTAGA ATTCCTGCAGCC	[47]
Thrombin detection at sub-nanomolar concentrations in diluted human serum, using two thrombin aptamers	0.1 nM	[48]	**Aptamer TBA29 (heparin-binding exosite)** AGTCCGTGGTAGGGCAGGTGGGGGTGACT	[49]
			Aptamer TBA15 (fibrinogen binding exosite) GGTTGGTGTGGTTGG	[50]
Bovine catalase detection in milk samples for food safety monitoring	20.5 nM	[51]	**Aptamer CAT1** CTTCTGCCCGCCTCCTTCCGACCTAGCAGTGGA CATGTGGCAGGGTGAAGTGGCATCGTCGGAG ACGAGATAGGCGGACACT	[52]
B-type natriuretic peptide detection, a biomarker for cardiac failure	1 nM	[53]	**Aptamer 8-12** TAAACGCTCAAAGGACAGAGGGTGCGTAGGA AGGGTATTCGACAGGAGGCTCACA	[54]
C-reactive protein detection in spiked human serum	43 aM	[55]	The CRP specific aptamer of the ref [55] was acquired from OTC Biotech. However, there is another aptamer sequence in the recent literature for CRP, as provided below:	
			Aptamer CRP-40-17 (truncated CRP-80-17) CCCCCGCGGGTCGGCTTGCCGTTCCGTTCGGC GCTTCCCC	[39]

4. Contemporary Techniques for Aptamer Selection

4.1. Robotic SELEX

The major advantage of the robotic method is the automation of time-consuming repetitive cycles, which ultimately allows robust, reproducible and parallelized selection of the aptamers for even multiple targets. Manual operations required are the preparation of the reagent solutions and the gel electrophoresis following the automated cycles. Immobilization of the target, incubation of the pool with the target molecule, washing and elution steps, PCR, RT-PCR, *in vitro* transcription, as well as the single-stranded DNA preparation steps, can be executed automatically. The automated systems usually accommodate a liquid handling station integrated with a thermal cycler, which can be controlled with an in-house or commercial software, as shown in **Figure 2**. Additionally, cooling units, magnetic separation units or vacuum systems can be combined with the existing platform to manipulate even more steps such as DNA purification or labelling.

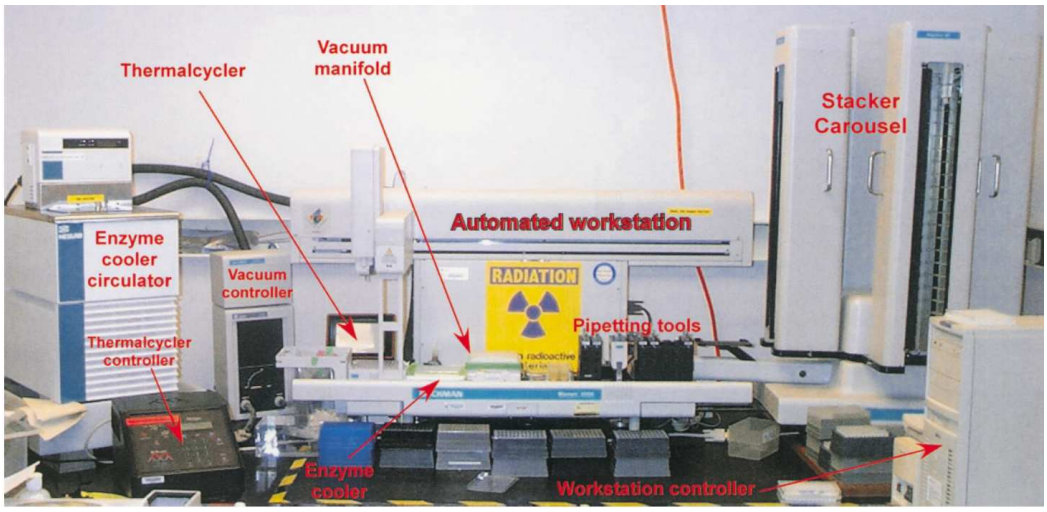

Figure 2. A photograph of the entire automated in vitro selection system by Cox et al.[56] Copyright © 2001, Elsevier

Recent progress in the automated systems has reached a level that a large number of proteins can be targeted within a course of months. Besides, the automation of *in vitro* translation might enable simultaneous selection of aptamers against the translation products of genomes,[57] which remains to

be seen whether it can be done in practice. **Table 3** presents a short list of aptamers produced using the automated selection platforms along with the automated SELEX steps.

Table 3: Examples of some aptamers selected using the automated selection platforms

Target	Automation Units	Automated steps	5'-aptamer-3' and K_D	Ref
HPV16 E7 oncoprotein specific aptamer-transfected cervical carcinoma cell lines disclosed induction of apoptosis[58]	Automated liquid handling system integrated with PCR machine and the magnetic separator segment	Incubation Elution Reverse transcription PCR *In vitro* transcription	**Aptamer A2** CCCUUCAUCAUUAACCC GUCCACGCGC (107 nM)	59
As a proof of the concept, aptamers against Lysozyme, wine fining agent, were selected using a wine simulating buffer during the selection	Robotic magnetic separator (FISHing) and solid phase emulsion PCR (BEAMing)	Incubation, washing and elution steps are fully automated and named as FISHing step while the amplification and ssDNA production steps were combined into one step called BEAMing.	**Aptamer LysApt2** CATCCGTCACACCTGCTC TTCAACGATTCTTTTTTTT TTGTCACGTTCGCATTGT CTTGGTGTTCGGTCCCGT ATC (4 nM)	60
Anti-lysozyme aptamers that function as efficient inhibitors of cell lysis	PCR machine, magnetic separator and filtration plate device along with a vacuum manifold system for actual selection step	Incubation Elution Reverse transcription PCR *İn vitro* transcription	**Aptamer Clone 1** ATCAGGGCTAAAGAGTG CAGAGTTACTTAG (31 nM, dissociation constant increased to 65 nM when the target was biotinylated.	56
Aptamers were selected against *in vitro* translated human U1A, a component of the nuclear spliceosome	Automated liquid handling system integrated with a PCR machine, magnetic separator, and filtration plate device	Incubation Elution Reverse transcription Amplification *In vitro* transcription and translation	**Aptamer UIA-R18C02** GGGAAUGGAUCCACAU CUACGAAUUCAAGCGC UGAGGUUGAGAACAUU GCACUACGUUCACUGC AGACUUGACGAAGCUU (4.5 nM)	57

4.2. Microfluidic-based SELEX

The incorporation of the microfluidic systems into the aptamer selection platforms has further revolutionized the SELEX by enabling more labour free and high throughput selections, as discussed in this section. Microfluidic technology is developed with the aim of miniaturizing biomedical testing systems, which has currently established superior performance to its sizeable equivalents. Manipulation of the microfluidic devices for the rapid and efficient aptamer selection has showed remarkable advantages over conventional methods, as well described by Lin et al.[61] Since the method is mainly processed on a chip, it enables robust aptamer selection on a small scale. Integration of magnetic particles into the microfluidic devices has also improved the sensitivity and the specificity of the screening processes. In addition, Peltier technology has enabled fast heating and cooling on the chip surface for thermal reactions like Polymerase Chain Reaction. For example, Botulinum Neurotoxin type A aptamer was obtained only after a single round of selection using the Continuous-flow Magnetic Activated Chip-based Separation device, (CMACS) designed by Lou et al.[62] The selection of C-reactive protein (CRP) specific aptamers was again performed automatically using a microfluidic chip system that was combined with the CRP modified magnetic beads.[63] More recently, Hung at al.[64] has designed and fabricated an automated microfluidic device employing magnetic beads for the high throughput screening of the multiple cell lines to competitively select aptamers against different types of the histologically classified ovarian cancer cells. The sophisticated device, as shown in **Figure 3**, required only five rounds of SELEX to generate aptamers against four different cell lines with nanomolar affinities. The completion of one round took only three hours offering a robust methodology compared to the conventional counterparts.

As in all automated selection methods, there is a cross contamination risk in microfluidic-based aptamer selection systems. In order to surmount the cross contamination problem, Lee et al.[65] proposed a microfluidic platform operating pneumatic valves to allow the sequential loading of samples, parallel incubation of oligonucleotides with five different target proteins that are entrapped in sol-gel spots to enable contamination free elution of the binding molecules. When eluting the bound molecules, cross contamination is avoided by sealing the elution chamber's pneumatic microvalves. Unlike the previous studies on the microfluidic selections employing magnetic beads for target binding, Lee and the colleagues applied sol-gel encapsulation strategy where the

oligonucleotides were diffused from the nano-porous structure to reach the targets, allowing multiple targeting.

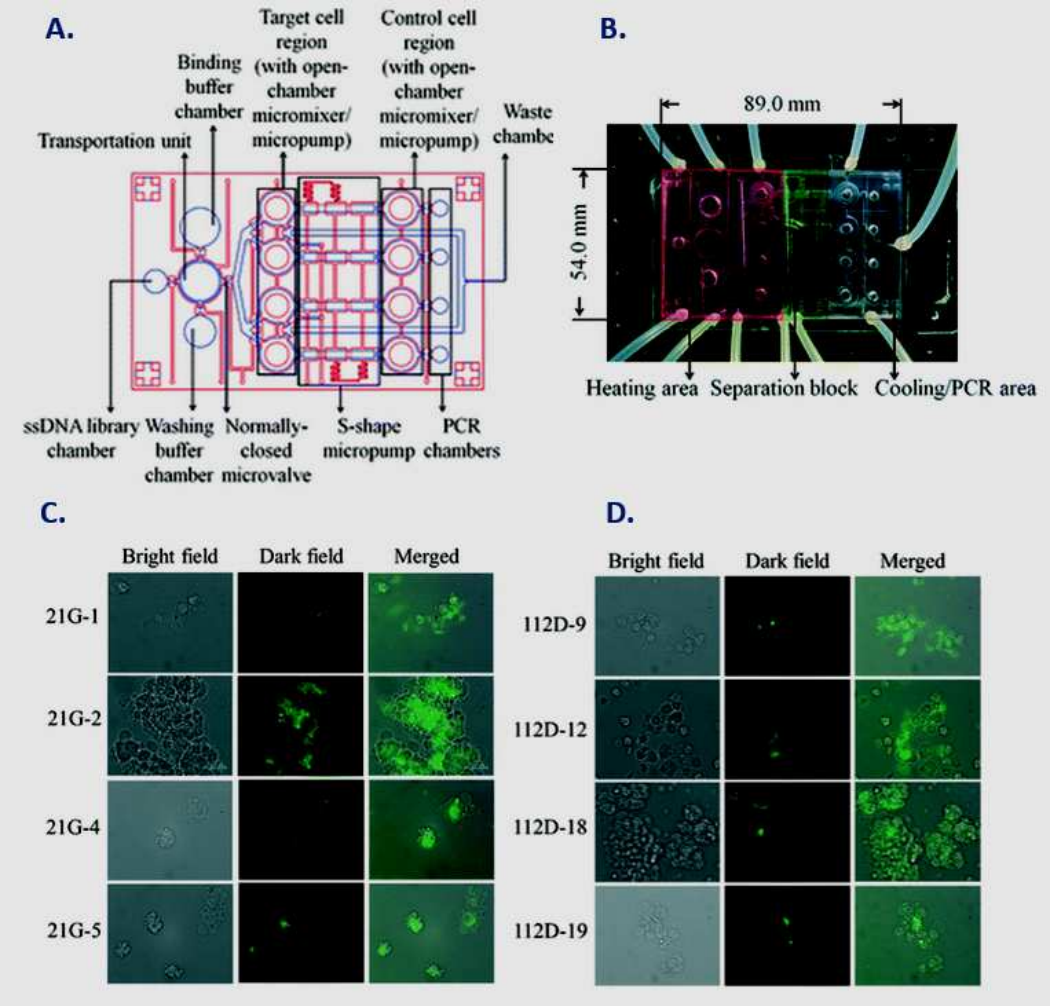

Figure 3. Automated microfluidic device fabricated for high throughput aptamer selection. A) Illustration of the instrument equipped with multiple micro devices including three reagent loading chambers (for ssDNA library, washing buffer and binding buffer), a transportation unit, closed microvalves, open-chamber micro mixers/micro pumps, serpentine-shaped (S-shaped) micro pump and PCR chambers; B) Photograph of the chip placed on top of a double temperature control module with a "separation block" to insulate against thermal conduction; red defines the heating area, green defines the distance block, and purple represents the cooling area; C) Fluorescence images of cell line TOV21G, stained with specific aptamers; D) Fluorescence images of endometrioid type cell line TOV112D, stained with specific aptamers. Reproduced from ref.[64]

Table 4 presents a couple of aptamers obtained after a few rounds of selection (maximum 6 rounds) using the microfluidic selection methodology. Overall, both the magnetic bead-based and the sol-gel spot based microfluidic screening systems offer a considerably rapid selection procedure. Additionally, microfluidic devices can facilitate multi-target selection in parallel that makes the technique superior to all the previous methods. Nevertheless, the construction of this type of sophisticated devices requires professionals in the field as well as competent and dedicated infrastructures, which limits their wider application.

Table 4: Examples of aptamers selected by the integrated microfluidic chip-SELEX technique

Analytes	5'-aptamer sequence-3' and K_D	Ref
Glycated hemoglobin (HbA1c) and the total hemoglobin (Hb) specific aptamers for the diagnosis, monitoring, and risk assessment of diabetes	**HbA1c aptamer** GGCAGGAAGACAAACACATCGTCGCGGCCTTAGGAGG GGCGGACGGGGGGGGGCGTTGGTCTGTGGTGCTGT (7.6 ± 3.0 nM) **Hb aptamer** GGCAGGAAGACAAACACCAGGTGAGGGAGACGACGC GAGTGTTAGATGGTAGCTGTTGGTCTGTGGTGCTGT (7.3 ± 2.2 nM)	66
Aptamer selected against influenza A virus, InfA/H1N1; a virus causes high, morbidity, mortality and even severe global pandemics	**InfA/H1N1 specific aptamer** GGCAGGAAGACAAACAGCCAGCGTGACAGCGACGCGT AGGGACCGGCATCCGCGGGTGGTCTGTGGTGCTGT (55.14 ± 22.40 nM)	67
Myoglobin (Myo) as an early marker for acute myocardial infarction; Human serum albumin, Bovine serum albumin and CRP used as the counter targets during the selection	**Aptamer Myo40-7-27** CCCTCCTTTCCTTCGACTAGATCTGCTGCGTTGTTCCGA (4.93 ± 0.43 nM)	68
Aptamer selected against Alpha-fetoprotein (AFP), a biomarker for liver cancer	**AFP specific aptamer** GGCAGGAAGACAAACAAGCTTGGCGGCGGGAAGGTG TTTAAATTCCCGGGTCTGCGTGGTCTGTGGTGCTGT (2.37 nM)	69

4.3. Next generation sequencing-based methods

High throughput Next Generation Sequencing (NGS) platforms have been used in the SELEX for a comprehensive analysis of the enriched aptamer pools. Identification of candidate aptamers based on their consensus sequences and the secondary structures is considered as a fundamental change in the field. In contrast to the NGS platforms where millions of sequences can be readily acquired from the enriched pools, only a few hundred clones are sequenced in the conventional SELEX platforms in order to identify the high-affinity aptamers. Aptamer candidates can be determined by the high-throughput sequencing at much earlier cycles of a selection starting from the naïve/non-enriched pool itself whereas the cloning based aptamer identification obliges evolution of the selection pool down to a few sequences. Based on the efficiency of the NGS platforms in terms of the read number and read lengths, almost a hundred million sequences can be obtained from an enriched pool, which offers a great deal of information on the enrichment level of the pool as well as the structural features of the individual sequence families. For instance, Wilson et al.[27] recently reported bivalent thrombin aptamers selected at single cycle using 454 GS FLX NGS platform that enabled comprehensive data analysis obtained from the sequential dissociation rounds. A counter selection step of 24 hours was followed by a bead-based target incubation (thrombin) step of 48 hours and a long dissociation phase of 96 hours. The dissociation step was realized in ten consecutive cycles where the supernatant was collected after each dissociation step and subjected to PCR, followed by the sequencing and data processing with 454 GS FLX software, MEME, Clustal Omega and mfold. As presented in **Figure 4**, the data displayed a gradual enrichment of the pool throughout the dissociation cycles, which reveals the high potential of the NGS in simplifying the selection of aptamers. By using the same NGS platform combined with the capillary electrophoresis based-SELEX of six rounds, Jing et al.[70] generated rhVEGF$_{165}$ specific aptamers with binding constants around 30 nM. It is important to note that aptamers selected depending on their abundance (read count or copy number) ratio and the consensus motifs, showed almost similar K_D values with the aptamers arbitrarily chosen from the round 4 of the same pool (with low abundance), indicating that high abundance ratio does not necessarily reveal aptamers with the highest affinities.

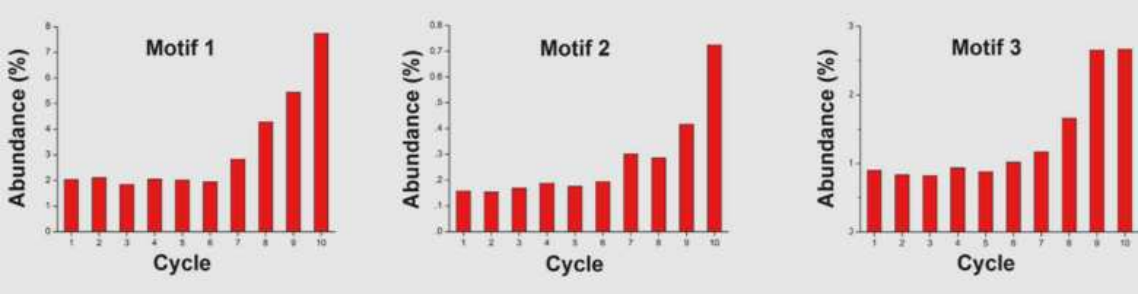

Figure 4. Bar graphs are showing enrichment of motifs 1–3 in each dissociation cycle of single-step NGS based selection. Reproduced from open-access ref.[27] Copyright © Wilson et al. 2014

In theory, however, the high abundance rate is expected to be in correlation with the pool enrichment as long as the bias originating from the selection, PCR, sequencing and the data processing is negligible. Consistent with the theory, Bawazer et al.[71] implemented the sequence similarity (motifs or consensus sequences) and the high abundance rate of the sequences in the aptamer selection, in which aptamers specific to zinc oxide (ZnO) semiconductor mineral surfaces (bio-mineralizing DNA aptamers, facilitating ZnO mineralization from a hydrated Zn(NO3)2 precursor) were selected only after one selection cycle using AB SOLID Sequencing platform and a novel in-house population clustering algorithm, called Auto SOME.[72] The authors selected a small proportion of unique sequences from the pre-processed NGS data, and eventually, the binding kinetics of the sequence motifs were found consistent with the abundance ratio of the chosen sequences, meaning the highest affinity aptamers were evolved from the most abundant sequence family. Except from the abovementioned sequencing techniques, Illumina sequencing platform has been the one used most frequently in SELEX due to the considerably higher number of overall sequencing reads and read lengths (number of bases sequenced/read per fragment).[73]

Although the next generation sequencing platforms have significantly expanded our horizons in aptamer selection, the processing of the massive data sets produced by the sequencing platforms has remained as a challenge because the software or online tools currently available cannot handle this much information in a reasonable timescale. Therefore, researchers often treat only a portion of the data chosen from the total pool that is projected to be representative for the whole data set. In the current state, enriched pools are processed using the tools such as AliBee,[40] Clustal W,[70] Clustal Omega,[27] MEME ChIP,[74] mfold,[75] RNAfold[76] or some local algorithms.[77]

Aptaligner algorithm has been recently introduced as new tool for the alignment of the vast selection pools containing around 5 million sequences, as detailed in the work by Lu et al.[78] Likewise, another algorithm called AptaCluster,[79] which is designed as an extension to the previous motif algorithm AptaMotif[80] by the same research group, has been reported to accommodate around 20 million sequences without loss in performance, implementing a procedure that enables the tracing of clusters over consecutive SELEX rounds, as presented in **Figure 5**.

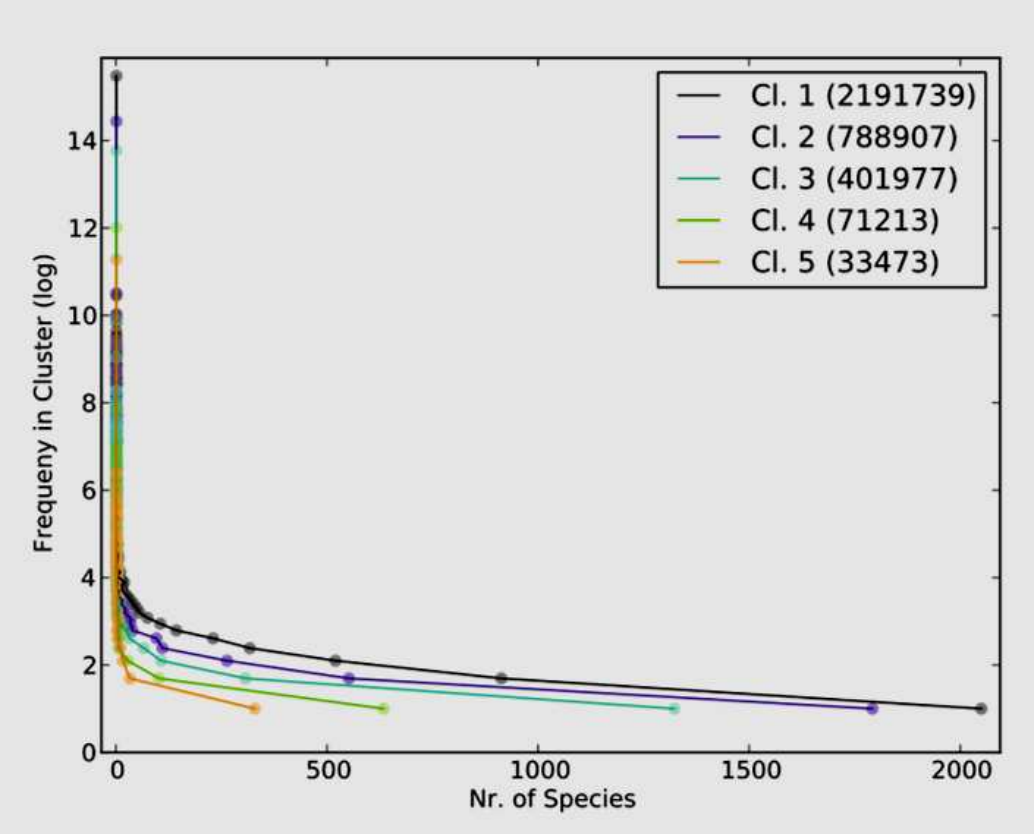

Figure 5. The frequency distribution of the members of the 5 largest clusters. The cluster sizes are given in the brackets. Reproduced from ref.[79] Copyright © 2014, Springer International Publishing, Switzerland

4.4. Other SELEX methodologies

In addition to the single selection methods mentioned above, there are other techniques that combine the existing selection strategies to produce high-affinity aptamers in a faster manner while maintaining the target affinity as high as in the previous techniques. For example, a Quantitative Parallel Aptamer Selection System (QPASS) that integrates the microfluidic selection and the next

generation sequencing techniques with in situ-synthesized aptamer arrays has been recently developed by Cho et al.,[81] who screened the binding affinities of around 15.000 aptamer candidates simultaneously for a given target using the microarray technology. An immediate illustration of the QPASS platform was presented in **Figure 6**. Because of the limitations in microarray chips, which can hold only a limited number of oligonucleotides at once (~$1x10^4$), the authors first enriched the start pool ($6x10^{14}$ oligonucleotides) using a microfluidic SELEX methodology for a few rounds, during which a relatively moderate selection condition was applied to conserve the sequence diversity. Following the NGS step, the whole data set was filtered based on the most repeated sequences from each SELEX round that were subsequently used for the microarray design along with the control sequences. The microarray was further used to measure the binding affinity of all the selected sequences in parallel, which yielded over a dozen aptamer candidates against cancer biomarker Ang2 with K_Ds around 20 nM. Although it allows only one target selection at a time, unlike some robotic[57] and microfluidic techniques[64] that could facilitate multiplex selections, QPASS system seems to be one of the fastest methodologies considering the massively parallel measurement of the binding constants from moderately enriched pools and the ability to introduce counter selection steps.

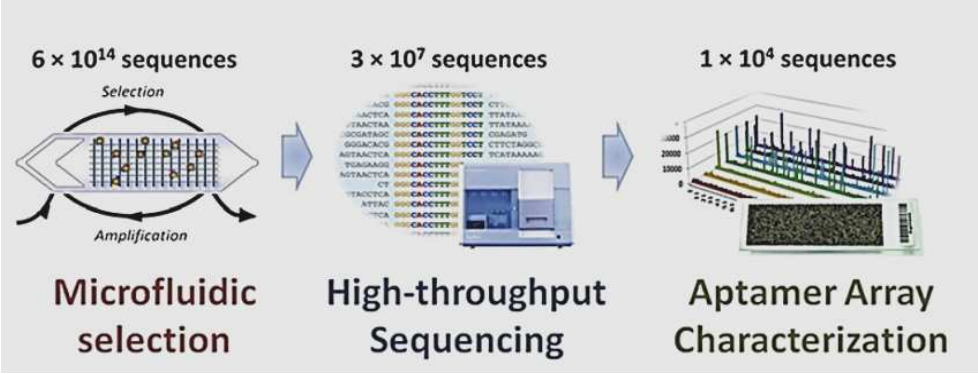

Figure 6. Overview of the QPASS platform. Aptamers were selected against a cancer biomarker, Ang2 in four rounds starting with around 1 nmol of the random library (~6×10^{14} sequences) (Left). The enriched pools were sequenced using Illumina GAIIx NGS instrument, obtaining ~3×10^7 sequences that were subsequently analysed for the sequence repeat number and the homology (Centre). A microarray chip was designed for the NGS data, which accommodated ~15,000 aptamers including eight identical in situ-synthesized aptamer arrays in which each of the 235 top candidate aptamers from every selection round and also control sequences were all represented in triplicate (Right). Reproduced from open-access Ref.[81] Copyright© 2013, Proceedings of the National Academy of Sciences, USA

Alternatively, there is Genomic SELEX which is quite similar to the conventional SELEX techniques regarding to the experimental procedures except for the start pool consisting of 200-300 base pair DNA molecules from the total genomic DNA of the projected cell. Many of the reports focused on the Genomic SELEX have been observed to be targeting transcription factors of the specific cultures in order to disclose their regulations within the cells like *Escherichia coli*. [82–84] The realization of the genomic SELEX requires a good quality of start library,[85] which is ideally representative of the genome of interest, and the genomic inserts that are proportionally included in the library. It is also possible to use the sheared genomic dsDNA as the input library.[86] Further experimental details and characteristics of the genomic SELEX technology have been discussed by Lorenz et al.[87] and Zimmerman et al.,[88] respectively.

5. Major Application Fields of Aptamers

5.1. Biomarker discovery and diagnostics

A number of modified SELEX techniques can be used to identify unknown biomarkers of the targets, accelerating the disease diagnosis, development of novel therapeutics and the detection platforms. High throughput biomarker discovery with SELEX was achieved by Larry Gold and co-workers who discovered around 60 potential biomarkers of the chronic kidney disease (CKD), two of which were already known CKD biomarkers.[89] The achievement of the authors is based on a new class of aptamers, called Slow Off-rate Modified Aptamers (SOMAmer), comprising modified nucleotides that mimic amino acid side chains. Additionally, the authors developed a novel SELEX strategy that only allows the selection of aptamers with slow dissociation constants. Multiplexed technology enabled by the SOMAmers has been used to discover biomarkers for early diagnosis of lung cancer,[90] malignant mesothelioma,[91] age-related neurodegenerative diseases[92] and recently Alzheimer's disease.[93] In another study by Webber et al.[94] slow off-rate aptamers were used to identify exosome-associated biomarkers, which revealed biomarkers that were previously associated with cancer exosomes, and also other biomarkers that are poorly correlated with the exosomes. Besides, the authors achieved to describe over 300 protein biomarkers of unknown association with prostate cancer exosomes, demonstrating the immense potential of aptamers in biomarker discovery. Likewise, Hung et al.[64] developed an integrated microfluidic-based SELEX technique to select slow

off-rate aptamers for high-throughput screening of the multiple cell lines, aiming early diagnosis of ovarian cancer.

AptaBID system (Aptamer-facilitated biomarker discovery), on the other hand, targets the biomarkers which are differently expressed on the cell surface.[95] Using AptaBID technology, the authors revealed DNA aptamers that specifically target mature and immature dendritic cells, moreover, the selected aptamers were used to isolate the novel biomarkers from the dendritic cell surfaces that were identified through mass spectrometry analysis. Unlike traditional biomarker discovery techniques, usually based on western blotting, mRNA screening or two-dimensional gel electrophoresis, AptaBID allows exponential detection of the cell surface biomarkers through multi-round aptamer generation strategy that reduces false positive signals by suppressing the stochastic variations in cell population. Another method called subtractive cell-SELEX[96] revealed seven aptamers that specifically bind to highly metastatic colorectal cancer LoVo cells with binding constants in the nanomolar range. Among those seven, the receptor binding aptamer W14 was used to deliver doxorubicin (drug) into the cells while the aptamer W3 (non-receptor binding aptamer) was conjugated with fluorescent nanoparticles (quantum dots) for targeted imaging applications.

Aptamers have also found significant applications in the diagnostic assays which currently being dominated by the antibody-based assays. The well-known diagnostic assay, Enzyme-linked Immunoadsorbent Assay (ELISA), for example, utilizes antibodies and the colour change reaction to identify the presence of substance. In the aptamer version of ELISA, named as ALISA,[97] Vivekananda and Kiel employed the biotinylated aptamers as the recognizing agents for tularemia antigen, in which the aptamers find their target immobilized on a filter and produce a colour change upon binding to streptavidin-conjugated horseradish peroxidase. Tularemia is a severe disease that can be acquired by humans through the infected animals such as cats, dogs or squirrels. *Francisella tularensisis* being the source of the tularemia disease has recently taken considerable attention due to its potential use as a biological welfare. With the aptamer modified ELISA sandwich assay, Vivekananda and Kiel reported a detection limit of 25 ng for the tularemia bacterial antigen, which is claimed to be superior to the ELISA in terms of the detection efficiency. Aptamer-tethered enzyme capture assay (APTEC), on the other hand, was developed for the rapid diagnosis of malaria from the clinical blood samples.[98] By using a previously reported DNA aptamer specific for *Plasmodium falciparum* lactate dehydrogenase,[99] the authors diagnosed malaria with a limit of detection value of 4.9 ng ml^{-1} that is within the clinical detection range since the malaria patients typically have the

enzyme at microgram levels. Unlike the ALISA or other colorimetric assays, APTEC does not require further reagents (etc. antibodies, nanoparticles) or laboratory equipment for disease confirmation as it gives an intense colour development that could be seen by naked eye.

Among many others, a particular attention has been also paid to the viral disease diagnosis through the aptamers, which is recently reviewed by Wandtke et al.[100] As listed in **Table 5**, aptamers have been selected against varies viral targets and that can be employed in diagnostic platforms in near future.

Table 5: Examples of aptamers selected against viral targets

Target	Method	5'-aptamer sequence-3'	Ref
Recombinant hemagglutinin 1 (HA1) protein from H5N1 Influenza virus, the major immunogenic surface glycoprotein	Affinity chromatography-based *in vitro* selection, using Ni-NTA superflow	**Aptamer 10** GAATTCAGTCGGACAGCGGGGTTCCCATGCGGA TGTTATAAAGCAGTCGCTTATAAGGGATGGACG AATATCGTCTCCC	101
Purified HA protein from influenza virus AIV H5N1 as well as the entire virus	Nitrocellulose acetate membrane/filter based SELEX	**Aptamer sequence 2** GTGTGCATGGATAGCACGTAACGGTGTAGTAGA TACGTGCGGGTAGGAAGAAAGGGAAATAGTTG TCCTGTTG	102
Whole subtype C HV-1_{CAP45} (human immune deficiency virus type-1) enveloped pseudo virus surface glycoproteins; gp120 $_{CAP45}$ and $gp41_{MN}$	Filter membrane based-SELEX using Nanosep 30 nM membranes	**CSIR1.1 ($gp120_{CAP45}$ binding RNA aptamer)** TAATACGACTCACTATAGGGAGACAAGACTAGA CGCTCAACCCCGAAGATGCCTTCATGAGCGCACC ATCTTGCCACCGACTTCAAATTCGACATGAGACT CACAACAGTTCCCTTTAGTGAGGGTTAATT **CSIR1.2 ($gp41_{MN}$ binding RNA aptamer)** TAATACGACTCACTATAGGGAGACAAGACTAGA CGCTCAACAGGACCGAGAGATGCAACTAGTGAT TTCCCTCATAATCATTGTAAGAGCTTCGACATGA GACTCACAACAGTTCCCTTTAGTGAGGGTTAATT	103
Aptamers distinguish between H1-HA1 protein and H5-HA1 protein from influenza virus, yet did not block the sialic acid-binding region of HA1	Glutathione agarose beads-based Counter-SELEX was applied using H5-HA1 as the counter target	**Ap1:**GGGTGGGTGGGAGGGGGTGGAGGTTGGG GGTTGGACGCAGAGTGC **Ap2:**TCGTGTGGGGTGGGTGTTGTGGGGGTGGG TGGGTGGGCTCGGCG **Ap3:**CGGTGGGCAGGAGGGGGTGGGCGGGTGG GGGGTCGCAATAGCGTC	4

Sialic acid receptor of HA1, SAR aptamers inhibit H1 hemagglutination at a low picomole dose	Epitope specific SELEX (two selection routes, HA1 targeting and SAR targeting, were performed using His-Tag affinity columns	**Aptamer CP9P536** TCCCTACGGCGCTAACCGATAGGTGTAGCGTGG GGCACATGTTCGCGCCACCGTGCTACA AC **Aptamer CP9P536 minimal binding region** GCGTG GGGCACATGTTC)	104
Hepatitis C virus Envelope protein, E1E2	Not clarified enough	**E1E2-1** GGTATGTGGAATAATCTAGCACCTACC **E1E2-2** GCAGTGTGGAATAATCTAGCACCCTGC **E1E2-3** CAGGAGGAATAATCTAGCTCCTG **E1E2-4** CAGGTCGATTAATCTAGGACCTG **E1E2-5** CACGTCGATTAAGATTGGACGTG **E1E2-6** CACGTCTATTAAGATTGGGACGTG	105

5.2. Aptamer conjugates for targeted delivery and imaging applications

Safe and efficient delivery of drugs during the treatment of cancer or infectious diseases have remained as a significant challenge for clinical translation and the development of new therapies due to the lack of safe and the reliable delivery methods. Drug conjugated antibodies (ADC) have been the achievement that made a considerable progress in clinical studies, some of which have already been approved by FDA (etc. Brentuximab vedotin, trade name Adcetris) while many others are under clinical development stage.[106] By virtue of the small size, stable structure with ease of synthesis and the low immunogenicity, aptamers have gained attention in the construction of targeted drug delivery systems in order to improve the efficacy.[107] Aptamers are considered as tumour-specific antineoplastic agents that could deliver the drugs directly into the cancer cells to inhibit the tumour cell growth and dissemination during the cancer treatment.[108] To date, aptamers have been conjugated with polymers like poly (ethylene glycol),[109] nanomaterials including the gold nanoparticles,[110] chitosan nanoparticles,[111] carbon nanotubes[112] for especially targeted drug delivery

purposes. The recent progress in the cancer therapy via aptamers have been summarized by Sun et al.[113] It should be noted that the conjugation of aptamers with the nanomaterials increase the nuclease resistance of the oligonucleotides as well as preventing them from the renal filtration due to the increased size, whereas the antibody usually conjugates do not such limitations owing to their size and better pharmacokinetic properties.[114]

Aside from the drug delivery applications, the nucleic acid aptamers have been used as the carriers of microRNAs with tumour suppression functions where the conjugates reduced the growth of oncogenic receptor tyrosine kinase tumors,[115] suggesting a novel tool for the targeted delivery of microRNAs with therapeutic potential. Some examples of aptamers used in the targeted delivery applications are given in **Table 6**.

Table 6: Examples of the aptamers utilized in the targeted delivery applications

Aptamer	**5'-Sequence-3'**	**Ref**	**Conjugated material**	**Targeted cell**	**Ref**
Aptamer **sgc8** that can recognize protein tyrosine kinase 7 (PTK7)	ATCTAACTG CTGCGCCGCCGGGAAAAT ACTGTACGGTTAGA	[116]	antitumor agent doxorubicin (Dox)	CCRF-CEM cells (T-cell Acute Lymphoblastic Leukaemia, T-cell ALL)	[117]
RNA aptamer **J18** that can recognize the extracellular domain of Epidermal growth factor receptor (EGFR)	GGCGCUCCGACCUUAGUCU CUGCAAGAUAAACCGUGCU AUUGACCACCCUC AACACACUUAUUUAAUGU AUUGAACGGACCUACGAAC CGUGUAGCACAGC AGA	[118]	Gold nanoparticles (20 nm in dm)	A431 epidermoid carcinoma cells	[118]
TDO5 aptamer that is selected against Ramos cells (a B-cell lymphoma cell line) and probe the protein called Immunoglobulin heavy mu chain (IGHM)	AACACCGGGAGGATAGTTC GGTGGCTGTTCAGGGTCTCC TCCCGGTGA	[119]	Micelle (a lipid tail phosphoramidite with diacyl chains onto the end of TDO5, inserted with a PEG linker)	Ramos (CRL-1596, B-cell line, human Burkitt`s Lymphoma)	[120]

Aptamers that bind to O-glycan-peptide signatures on the surface of the breast, colon, lung, ovarian and pancreatic cancer cells. 5TR1 aptamer is specific to MUC1-5TR; 5TRG2 aptamer is specific to MUC1-5TR GalNAc (Tn antigen); GalNAc3 aptamer is specific to N-Acetylgalactoseamin (carbohydrate component)	**5TR1** GAAGTGAAAATGACAGAACACAACA **5TRG2** GGCTATAGCACATGGGTAAAACGAC **GalNac3** AAGGGATGACAGGATACGCCAAGCT	[121]	Photodynamic therapy agent, Chlorine e6 (Ce6)	MUC1^{+} Epithelial cancer cells that have O-glycan-peptide signatures on the surface unlike to the standard epithelial cells: T47D, MCF-7, PANC-1, BxPC3, A549, MGH13, OVCAR-3, HT-29	[121]
GL21.T RNA aptamer binds to extracellular domain of oncogenic receptor tyrosine kinase Axl and antagonizes it	AUGAUCAAUCGCCUCAAUUCGACAGGAGGCUCAC (The aptamer sequence was obtained by truncating a reported malignant U87MG specific aptamer, GL21)[122]	[123]	Conjugate with miRNA let-7g that has tumour suppressor function, inhibiting the growth of Axl-expressing tumours	Axl-expressing cancer cells (A549)	[115]

Given their suitability for conjugation with nanomaterials and fluorescent molecules, aptamers have been also used as the target specific probes for molecular imaging in place of the traditional contrasting agents. As comprehensively described in the review by Wang and Farokzhad,[124] aptamers have been successfully employed in optical imaging, anatomic or MR imaging, CT imaging, ultrasound imaging and nuclear imaging. Although there are several antibodies with high specificity towards their targets as well as long plasma half-lives, the immunogenicity and large spatial conformations prevent their utilization as the effective imaging probes, which pave the way for rational design of aptamers with higher clinical significance and longer plasma lives, as the future imaging and/or theragnostics probes. A short list of aptamers that have been used as the molecular imaging probes is presented in **Table 7**.

Table 7: Examples of the aptamers used as molecular imaging probes

Aptamer	5'-sequence-3'	Ref	Target cell lines	Scope	Ref
MUC 1 aptamer targeting Mucin 1 that is well known tumour marker present in a variety of malignant tumours	GCAGTTGATCCTTTG GATACCCTGG (Pegylated for higher tumour/normal tissue contrast ratio and faster clean rate, after which covalently attached to the hydrophilic near-infrared dye MPA)	[125]	The human cell lines: MCF-7 (breast cancer), A549 (non-small lung carcinoma), and HepG2 (hepatocellular carcinoma) *In vivo* tests were also performed using the tumour bearing, Hep2G, mouse models	Near-infrared fluorescent probe for malignant tumour imaging	[126]
AS1411 aptamer (formerly known as **AGRO100**, is **GRO29A-OH** with the 3 × 5' thymidines removed) targeting nucleolin, a multifunctional protein that is highly expressed by cancer cells, both intracellular and on the cell surface. AS1411 is known as a non-SELEX aptamer	TTGGTGGTGGTGGTT GTGGTGGTGGTGG (AS1411 is a non-SELEX aptamer)	[127]	Cancer cell lines, U-87MG, PC-12, F11, C6, HepG-2, SK-Hep-1, Caco-2, CT-26, TPC-1, NPA, U-2OS, HeLa, A549, PC-3 and F9 cells, and normal cell lines, MSC, F3, CHO, 293, L132 and TM-4 cells	Theragnostics probe consisting of AS1411 aptamer (for specific targeting), miRNA-221 molecular beacon (to inhibit miRNA 221) and the fluorescence magnetic nanoparticle (for imaging)	[128]
SYL3C aptamer (minimal form of SYL3), targeting Epithelial cell adhesion molecule (EpCAM or CD326) protein and the cells expressing EpCAM	CACTACAGAGGTTGC GTCTGTCCCACGTTG TCATGGGGGGTTGGC CTG	[129]	Specificity of the generated aptamers was tested using breast cancer cell line MDA-MB-231, gastric cancer cell line Kato III, and control cell line HEK-293T	Novel probes for targeted cancer therapy, cancer cell imaging, and circulating tumour cell detection.	[129]

5.3. Food safety and environmental monitoring

The contamination of foodstuff with chemicals like pesticides, toxins, veterinary drugs have caused serious health problems which, therefore, requires efficient systems for the continuous detection of the food content from production to the consumption. A number of aptamers have been developed against the toxic substances, and used in the construction of biosensors in order to monitor and control the possible hazards. A biosensor may consist of many parts in which the recognition part involves a component that detects the analyte with high specificity at low concentrations. Aptamers have been used as the recognition part in biosensors based upon the facts that they can be regenerated easily, they provide homogeneous preparation with negligible batch to batch variation, they provide thermal and chemical stability as well as labelling opportunities. To date a number of biosensors comprising aptamers (also called as aptasensors) have been developed for numerous analytes including the food safety-related targets such as antibiotics,[130] mycotoxins,[131] heavy metals,[5] pesticides,[132] some plastic polymer products used for food packaging[133] and foodborne pathogens.[32]

Antibiotics were usually added to animal's feed, especially to cow's feed, to promote animal growth and production of milk for dairy purposes. The assembling of antibiotics in food-producing animals, cows, caused antibiotic-resistant in humans. Thus, European Union Legislation on animal nutrition banned the use of antibiotics in animal feed for growth promotion. Currently, there are aptamers generated to detect antibiotics like chloramphenicol[130] or aminoglycosides like streptomycin[134] and kanamycin A.[135]

Mycotoxins, known as toxic micro fungal secondary metabolites, have been used as the antibiotics and growth promoters due to their pharmacological activities. The majority of the mycotoxicoses result from the consumption of contaminated foods where even trace concentrations of mycotoxins in food products may create health problems extending from disease to death.[136] The first mycotoxin DNA aptamer was developed against the most abundant food-contaminating mycotoxin known as Ochratoxin A (OTA),[137] which is produced mainly by many *Aspergillus species* and *Penicillium verrucose.* Fumonisin B1 is another member of the mycotoxins family that is usually produced by a number of *Fusarium* species, and it is considered to be involving in esophageal cancer.[138] Given its toxic effects through the contaminated corn-based crops, a number of analytical methods have been developed for the detection of Fumonisin B1 including the liquid chromatography and some other immunoaffinity assays. McKeague et al.[139] screened and characterized a number of DNA aptamers

that bind to Fumonisin B1, among which the aptamer called FB1 39 was shown to detect the analyte in nanomolar range, offering a robust reagent to construct biosensors that can be easily operated by the crop handlers in the field.

There are several reports confirming the presence of genotoxic and carcinogenic dyes like Rhodamine B, Sulforhodamine B and Melamine especially in the processed foodstuffs containing chili or curry powder, Curcuma and palm oil. These substances are usually added to the food products in order to increase the apparent protein content. Wilson et al.[140] developed a DNA aptamer targeting Sulforhodamine B with relatively low binding affinity in real samples (around 600 nm) while another study by the same group revealed an RNA aptamer called SRB-2 that binds to the same target with higher affinity, with a reduced binding constant of around 310 nM.[141] Given the anionic character of the fluorescent dye (Sulforhodamine B) due to the negatively charged sulphonate group, the discovered RNA aptamer is an unusual example of the anion detection by RNAs. Malachite green, then again, is a synthetic cationic dye used to colour cotton or paper-based materials. It has been used as an antifungal agent in the field of aquaculture in order to cure parasitic or fungal diseases in fish. However, it has been reported that Malachite green and a metabolite of Malachite green, known as Leucomalachite green, may cause toxic effects extending to gene mutations,[142] although there are conflicting reports. A malachite green aptamer (DNA, clone MG-4) with negligible affinity for the Leucomalachite green was developed in 1999[143] which was later characterized comprehensively by Stead et al.[144] who also developed a novel aptamer-based assay for the express detection and semi quantitation of Malachite green in fish tissue.

Heavy metals are another threat to the human health and the environment, because they can cause cancer and damage to the central nervous system. Human exposure to this metal group can occur through direct ingestion, such as direct water intake from the heavy metal contaminated soil or consumption of the crop products that are grown in heavy metal accumulated soil. Therefore, rapid detection and removal of the heavy metals from the contaminated areas have been one of the most leading research topics worldwide.[145,146] A high-affinity DNA aptamer was selected by Kim et al.[5] for the elimination of arsenic from Vietnamese groundwater, in which the aptamer Ars-3 was successfully applied to the field samples, where the arsenic concentrations ranging from 28.1 to 739.2 μg/L were removed after 5 min of incubation.

Bisphenol A (BPA), which has been used as one of the core monomers in common polymer plastic products since 1930s, is a potential hazard to foetuses, infants, and young children as stated in the

report by United States Food and Drug Administration.[147] Lee and co-workers developed an anti-BPA aptamer and aptamer-sandwich based carbon nanotube biosensor that could detect BPA at very low concentrations.[148] A DNA aptamer (called #3, R11) specifically targeting Bisphenol A was also developed by Jo et al.,[149] which does not recognize the Bisphenol B (one methyl group difference with BPA) unlike the Bisphenol A antibody, used in conventional Bisphenol assays like ELISA.

A list of aptamers that have been generated to target food contaminants, as well as the environmental substances, is provided in **Table 8.**

Table 8: Examples of the aptamers used in biosensor constructions

Analytes	Selection Method	Aptamer name, 5'-sequence-3' and K_D	Ref
Antibiotics Chloramphenicol	Tosyl-activated magnetic bead-based affinity selection	**Aptamer 7** ACTTCAGTGAGTTGTCCCACGGTCGGCG AGTCGGTGGTAG (0.76 uM)	130
Antibiotics Streptomycin	Streptomycin-coated epoxy magnetic bead-based selection	**Aptamer STR 1** GGGGTCTGGTGTTCTGCTTTGTTCTGTC GGGTCGT (199.1 nM)	134
Mycotoxins Ochratoxin A	Affinity column with immobilized Ochratoxin A was used in the selection	**Aptamer 1.12** TGGTGGCTGTAGGTCAGCATCTGATCG GGTGTGGGTGGCGTAAAGGGAGCATCG GACAACG (0.36 uM) **Aptamer 1.12.2** GATCGGGTGTGGGTGGCGTAAAGGGA GCATCGGCA (0.2 uM, aptamer 1.12.2 folded in a different buffer had a K_D of 50 nM)	137
Heavy metal Arsenic	Affinity resin based selection; 4-aminophenylarsine oxide was immobilized on an Affigel 10 resin	**Aptamer Ars-3** TTACAGAACAACCAACGTCGCTCCGGGT ACTTCTTCATCG (4.95 ± 0.31 and 7.05 ± 0.91 nM for As(V) and As(III), respectively)	5

Plastic monomer Bisphenol A	Bisphenol A immobilized epoxy-activated Sepharose 6B resin via hydroxyl groups	**Aptamer #3 (R11, from the round 11)** CCGGTGGGTGGTCAGGTGGGATAGCGT TCCGCGTATGGCCCAGCGCATCACGGGT TCGCACCA (8.3 nM)	[149]
Fluorophore Sulforhodamine B	Affinity chromatography based selection was applied using the pre-blocked Sulforhodamine b agarose column	**Aptamer SRB-2** GGAACACUAUCCGACUGGCACCUGUG CUCUAUAGCAGAAUGCUAACAUUAGA UGAUGGAGGGGCGCAAGGUUAACCGC CUCAGUACAGGUGCCUUGGUCAUUAG GAUCCCG (Ligand in solution: 310 nM, ligand immobilized: 70 nM) **Aptamer SRB-2 minimal sequence** GGAACCUCGCUUCGGCGAUGAUGGAG AGGCGCAAGGUUAACCGCCUCAGGUU CC (25% better binding affinity)	[141]
Dye Malachite green	Affinity chromatography based selection was applied using the Malachite green agarose, synthesized in-house.	**Aptamer MG-4 minimal binding sequence** GGAUCCCGACUGGCGAGAGCCAGGUA ACGAAUAGGAUCC (≤1 mM)	[143]

5.4. Aptamer conjugated nanomaterials

Practically, several factors such as the production cost, ease of manufacturing, stability under different conditions (e.g. non-aqueous solvents, high temperatures), help aptamers finding their way in the multidisciplinary research area where especially biotechnology interacts with the material science. Among many advantages of the aptamers over other recognizing agents, pre and post modification capability, has made aptamers versatile tools for the construction of bio-conjugated nanomaterials.

Unique properties of gold nanoparticles like tunable dimensions, electronic, optical and catalytic activity, high surface-to-volume ratio, stability, biocompatibility and ease of surface modification have made them excellent scaffolds for nanobiotechnology. Aptamer-conjugated gold nanoparticles, for example, have been tremendously used in sensing, to detect a variety of molecules from ATP[150] to urinary methylamphetamine,[151] at nanomolar concentrations. The thiol modification has been

considerably applied for the aptamer labelling, facilitating surface modification of the gold with oligonucleotides without sophisticated procedures. It is known that thiol groups assembled on the gold surface can survive up to 35 days under physiologic conditions,[152] which provides a significant advantage for field studies, as well as point of care devices based on the gold nanomaterials.

Carbon nanotubes comprise another application field for aptamers where they can non-covalently interact with the specific oligonucleotides through π-π interactions,[153] allowing their utilization as field effect transistors,[154] biosensors for non-polar small molecular species[148] or carriers for the targeted siRNA delivery.[112] Owing to their hollow structures formed by single-atom-thick sheets of graphene, carbon nanotubes possess a unique aspect ratio that is greater than any standard material, giving their characteristic features like exceptional mechanical strength, electrical and thermal conductivity. Dispersion of the carbon nanotubes has been a challenge for long because of the hydrophobic surface properties of the carbon nanotubes, preventing their effective utilization in several applications. Among many chemical substances (mainly surfactants) and biological agents (DNA and proteins) that have been used in the nanotube dispersions for sake of better quality, there are also peptide aptamers generated specifically for single wall carbon nanotubes which showed higher dispersal ability than the several organic surfactants reported previously.[7]

Modification of fluorescent nanoparticles such as quantum dots and upconversion nanoparticles with aptamers has laid the foundation of multiplexed detection based on the different emission wavelengths of the luminescent nanoparticles. For example, various types of upconversion nanoparticles that emit the light at different wavelengths were decorated with food pathogen-specific aptamers and utilized in multiplexed detection of three different pathogens simultaneously, [155] as it is illustrated in **Figure 7.** A similar method based on the multicolour quantum dots decorated with antibodies were shown earlier by Yang et al.[156] that had limitations due to the sensitivity of the antibodies. In the same context, an aptamer modified quantum dot assay was shown to be able detect zeptomole concentrations of C-reactive protein in spiked human serum,[55] showing the excellent sensitivity and stability of the aptamers. Unlike traditional fluorescent molecules, upconverting nanoparticles are excited with infrared light sources and they emit the light in the visible region, reducing any possible noise from the biological contaminants that usually emit the light in the UV-visible region. Moreover, the upconverting nanoparticles lack of autofluorescence that is what significantly increases the signal to noise ratio,[157] they have longer lifetimes and they can be produced at different emission wavelengths based on the elements used during the synthesis,[158]

allowing their integration into the multiplexed sensing platforms. Another striking application of the aptamer modified upconverting nanoparticles was the latent fingerprint detection in the fingerprint ridges through the lysosome specific aptamers,[159] as illustrated in **Figure 8.** The authors also showed the application of the method in cocaine detection using the cocaine specific aptamers, promising for future exploitation of the system for a wide variety of exogenous targets by only changing the aptamers.

Based on the conformational changes of the aptamers upon interaction with nanostructures like thin films, a biocompatible aptamer-polyelectrolyte film system was developed using a layer-by-layer approach.[160] Using fluorescence microscopy, the ability of the sulforhodamine B aptamer to bind its cognate target was demonstrated while sequestered in a chitosan-hyaluronan film matrix, showing the unlimited opportunities for smart material construction through the aptamers.

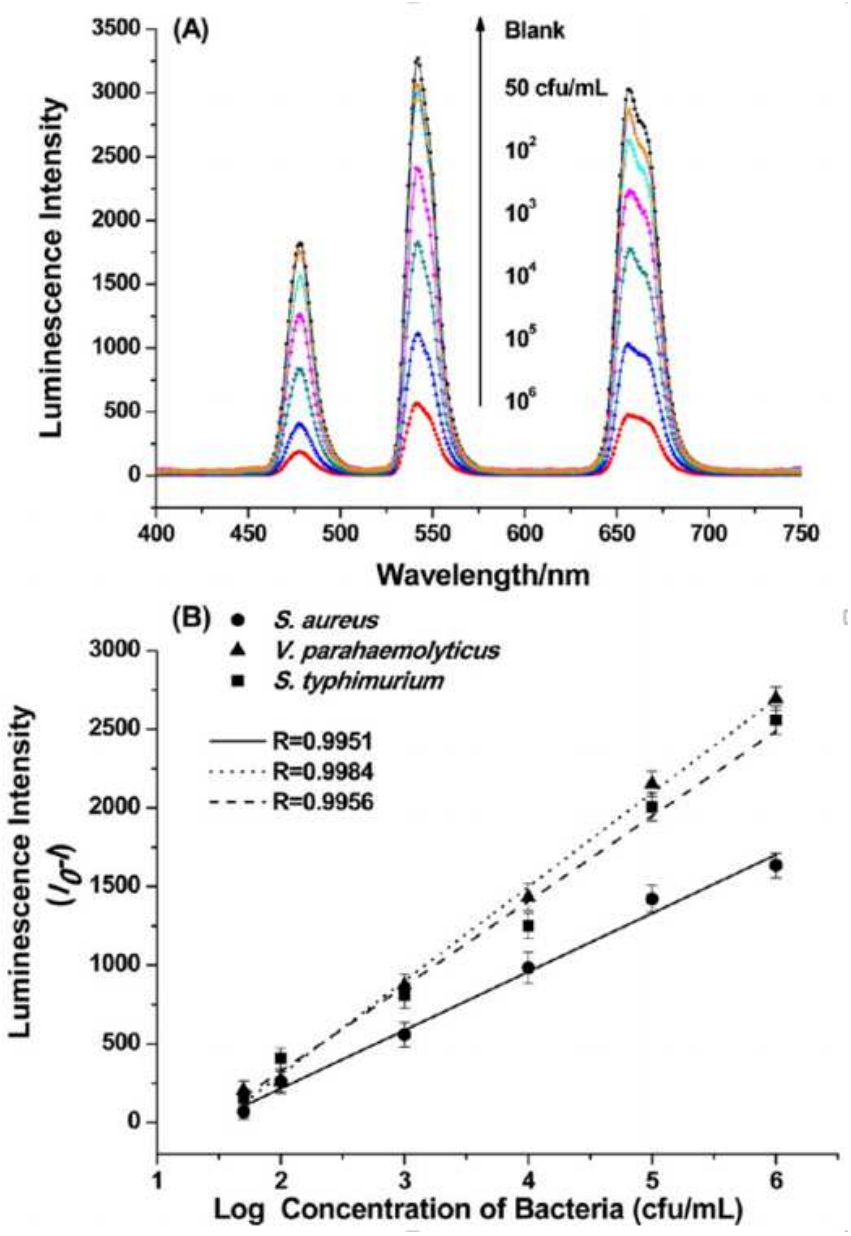

Figure 7. Simultaneous detection of three foodborne pathogen by the aptamer modified multicolour upconversion nanoparticle assay A. Change in the luminescence intensity of the aptamer modified upconverting nanoparticles upon introducing pathogens at different concentrations B. A standard curve of the corresponding upconversion luminescence intensity versus the concentrations of three bacteria Reproduced with permission from ref.[155] Copyright © 2014, American Chemical Society

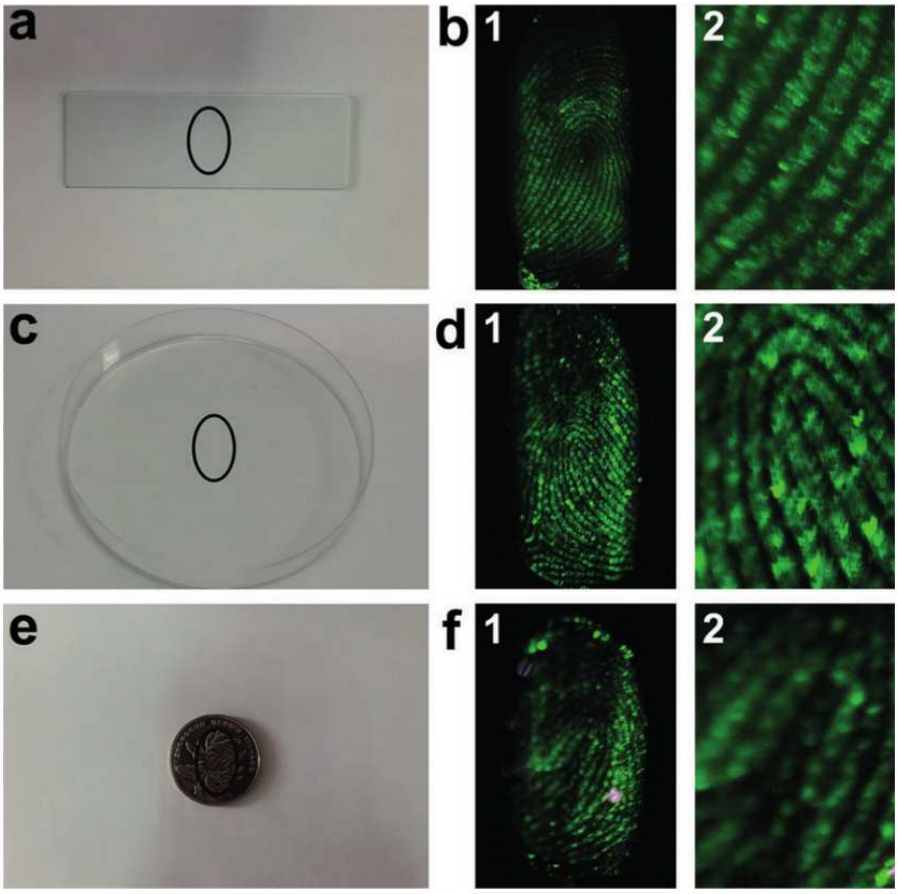

Figure 8. a) Photograph of a glass microscope slide with a latent fingerprint in the black circle; b) Luminescence image of fingerprint on the slide (1) and the corresponding magnified image (2); c) Photograph of a Petri dish with a latent fingerprint in the black circle; d) Luminescence image of fingerprint on Petri dish (1) and the corresponding magnified image (2); e) Photograph of a patterned coin with a latent fingerprint in the black circle; f) Luminescence image of the fingerprint on the coin (1) and the corresponding magnified image (2). Reproduced with permission from ref.[159] Copyright © 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

6. Challenges and Future Perspectives

It has been almost a quarter century since Andrew Ellington and Larry Gold revealed the aptamers as a new class of selected oligonucleotides rivalling antibodies. Since the inception, hundreds of aptamers have been selected from combinatorial oligonucleotide libraries, spanning a wide range of targets regardless of the size, surface charge, and toxicity, physical, chemical or biological structure.

Given their small size, *in vitro* screening capability, chemical and thermal stability, ease of labelling and the productiveness in relation to the overall synthesis cost, aptamers have been employed in a number of different fields including biotechnology, biomedical and environmental diagnostic, chemistry, material science and nanotechnology. However, in contrast to the rapid development and exploitation of the aptamers, there is only one aptamer clinically approved to date (Pegaptanib aptamer, or Macugen from Pfizer), and some others under various stages of clinical trials.[161,162] For example, PDGF-B aptamer (Fovista®) being developed by Ophthotech is now under phase IIa clinical trials to evaluate the efficacy of Fovista® 1.5mg along with the anti-VEGF drugs.[163] As discussed by Pei et al. earlier[164], nucleolin specific AS1411 aptamer (although it is not selected with the known SELEX methods) showed minimal toxicity in patients with advanced solid tumours, and the Phase II clinical trial is currently in progress with patients that developed metastatic renal cell cancer and AML. The latest developments regarding the clinical trials of AS1411 aptamer can be found in the report by Rosenberg et al.[165] According to the clinical trial information service provided by the U.S. National Institutes of Health, ARC1779 aptamer selected against the A1 region of von Willebrand factor (vWF) completed the phase II pilot study in 2008 (NCT00632242).[166] Although phase II clinical trials of the aptamer Nu172, selected to bind and inhibit thrombin, had been estimated to be completed in 2013 (NCT00808964)[167], no update is available regarding current situation of the aptamer Nu172. Aside from the ones under clinical trials, there is Pegnivacogin generated against Factor IXa (also known as anticoagulant, Revolixys™), which failed in the phase III clinical trial due to the severe allergic reactions that affected 3,232 patients participated in the trial.[168] A current list of other aptamers under clinical trial is lately provided by Zhou et al.[169]

Although the success in the clinical trials of the therapeutic aptamers seems currently limited, the growing academic and commercial interest in the field, promising data from the pre-clinical reports as well as the continuous funding may overcome the current limitations, and create a substantial progress in the coming years. As discussed earlier by Ni et al.[161] and Sundaram et al.,[162] the phosphate backbone of the oligonucleotides makes the aptamers susceptible to the nuclease degradation (either serum ribonucleases or exonucleases), significantly reducing their half-lives in serum. That is one of the challenges that aptamers face during the clinical trials. As mentioned earlier in this review, there is a number of chemical modifications available to make the aptamers resistant to the nucleases, in addition, there are polymer or nanomaterial conjugations available protecting the aptamers from the renal filtration. However, the cost of oligonucleotide modifications has to be

reduced for especially large scale manufacturing, enabling wider access to the end users. Another challenge in the clinical trials is the toxicity threshold,[114] the more aptamer go through the clinical trials the more toxicity assays must be handled beforehand since the current information about the potential toxicities (dose dependency, consequences of non-specific targeting, charge effect due to the anionic structure) of the aptamers is quite limited. Last but not least, there is an immense need for novel bioinformatics tools that are capable of processing the large-scale sequencing data along with the secondary structure predictions, in order to gain further insight into the correlation between the pool enrichment and the aptamer affinities. Although there is still a long way for the aptamers to go quickly from the laboratory benches to the markets, it is indeed a very interesting and promising field that is well worth the effort.

7. Conclusion

This report primarily focuses on the significant developments in the SELEX procedures that have enabled fast, high throughput, cost effective and the labour free selection of aptamers. We have also provided some major application fields of the aptamers where they have been used as the key components. Selection of aptamers with the highest sensitivity and selectivity for a broader range of targets, especially the targets with clinical importance, will be of interest to future researchers as well as the industrial bodies that are specialized in nano and biotechnologies. Investigating aptamer array-based schemes for the high-throughput detection platforms while discovering novel methods for multi-target parallel aptamer selection will have a significant impact on the development of next-generation field instruments, analytic tools and point of care diagnostics that employ aptamers as the core recognizing elements.

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