AQ1 LONG UN-INVITED REVIEW ARTICLE

Applications and modifications of aptamers: potential tool for medical microbiology

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AQ4 Aptamers are ligands made of single-stranded DNA, oligonucleotide RNA or short peptides which bind specifically to their target molecules with high affinity. They resemble different types of monoclonal, polyclonal and recombinant antibodies in their mode of attachment. Aptamers optimally bind their targets in a range of μmol to ρmol. Targets vary from small molecules to macromolecules to whole cells. Systematic evolution of ligands by exponential enrichment is an affinity-based screening method for aptamers. Examples of aptamer applications include structural analysis of molecules, affinity/specificity testing, epitope mapping, making aptamer libraries by systematic evolution of ligands by exponential enrichment, pathogenic targeting, specific molecular targeting, live cell targeting, analytical applications, therapeutic potentials and drug delivery. Staphylococcal enterotoxin B is one of the most important bacterial toxins in different disorders. Aptamers offer a fast, feasible, reliable and affordable method for detecting this toxin.

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AQ5 Introduction

Recent developments in molecular biology and bioinformatics have improved our expertise in molecular recognition and detection. Concurrently, adaptive molecular evolution assays (such as phage display) along with in-vitro detection technology and advances in bioinformatics have made it possible to narrow down the range of large datasets to make exact predictions in molecular recognition [1]. Designing aptamers is still in its infancy in biological determination because of the limitations of our knowledge about molecular structures. The word aptamer was coined first by Szostak *et al.* meaning 'to fit the part' ('apta' means 'to fit' in Latin and 'meros' means 'part' in Greek) [2]. There is a 25-year history of aptamer science [3]. Aptamers are single-stranded DNA (ssDNA), oligonucleotide RNA ligands which bind with high affinity specifically to their target molecules in the same fashion that different kinds of monoclonal, polyclonal and recombinant antibodies bind their targets [4]. Peptide aptamers also exist with similar properties [5]. Natural aptamers such as riboswitches can also be found among Reg-RNAs (a regulatory RNA motif). Aptamer-target binding affinity is in the range of μmol to pmol. They AQ6

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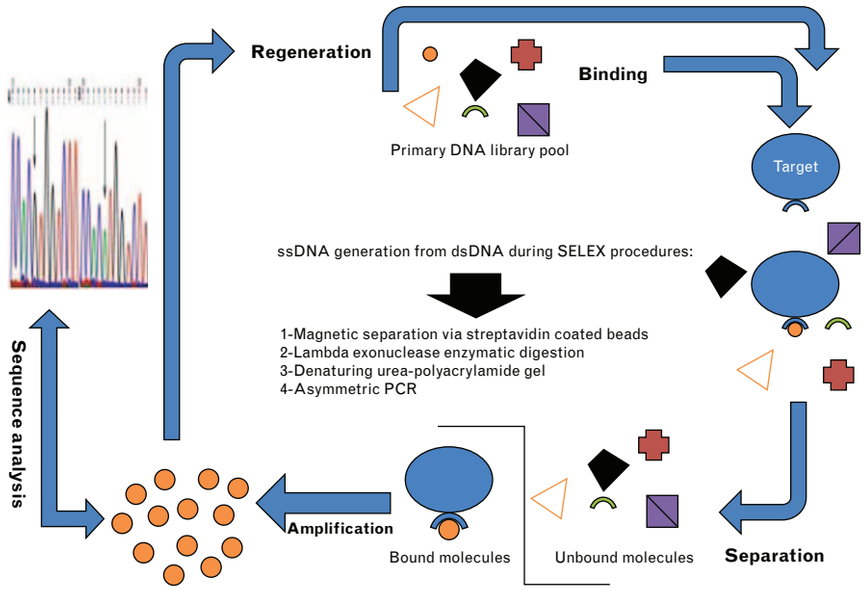

AQ22 **Fig. 1. Systematic evolution of ligands by exponential enrichment procedures and different methods which used to obtain single-stranded DNA from dsDNA.**

interact and bind to their specific target through three-dimensional structural recognition. The binding to the target is so specific that it makes aptamers able to discriminate between two similar molecules containing just two different functional groups or even the same molecules with different conformations [5–7]. The affinity and specificity of aptamers and antibodies are comparable (Table 1). Selected aptamers have been used *in vitro* for AQ7 various targets [40]. Targets range from small molecules to macromolecules to whole cells. In-vitro selection of aptamers has the potential of numerous chemical modifications, various conditions of selection and scale-up synthesis even up to kilogram scales. On the contrary, antibodies are developed and produced by in-vivo biological assays and their target antigens are almost limited to nontoxic macromolecules. Aptamers have superior properties compared with antibodies in target range, selection flexibility, postsynthesis modifications and in production costs. For example, aptamers can function both inside and outside cells [5,41], whereas antibodies usually interact outside cells to target secretory proteins or cell surface receptors. In therapeutic applications, aptamers have shown no intrinsic toxicity and immunogenicity [4,5,9,42,43]. Their capability as a potent inhibitory activity agent has been described in various studies and makes them an ideal candidate for therapeutic uses. Systematic evolution of ligands by exponential enrichment (SELEX) is an affinity-based selection method for aptamers [4]. Variations of the SELEX protocols have allowed isolation of aptamers with specific desirable properties expanding the repertoire of aptamer functions. Nucleic acid aptamers are typically isolated from large libraries containing approximately 10^{15} different sequences [1]. Various microbial and chemical toxins [1,44], organic and inorganic dyes [1,45], drugs, different proteins [1,46,47] and eukaryotic cells have been described as targets [48]. Obviously, aptamers are attractive molecular recognition agents compared with antibodies for therapeutic purposes, diagnostic means [48,49] and biosensing [49,50]. In addition, genomic SELEX are designed for genomic aptamers, which function on nucleic acid domains that identify and bind specific ligands [43,51]. Major sequence databases such as 'GenBank' [52,53], 'EMBL' [54,55] and 'DDBJ' [56] do not support the list of artificial aptamer sequences. Thus, some alternative databases such as 'Aptamer Database' designed for comprehensive sequence search for aptamers and nonnatural ribozymes generated by in-vitro selection methods have been used. This database is updated monthly and is available to the public at http://aptamer.icmb.utexas.edu/. In addition, there are some bioinformatics programmes such as 'ValFold' (free package available at http://code.google.com/p/valfold/) for computational analysis of aptamers [57]. Another free open licensed community-built source for functional and structural aptamer data is Aptamer Base (http://www.free base.com) that provides information for more than 22 million topics and its literature entries, which is accessible from 2006 to present, and data are added on a weekly basis. The focus of this database is on experimental designs for generating results that are used for identification of biomolecular interactions [58]. Aptamer biology and aptamers consist of some phrases, characteristics, modification and specific procedure by its own that could be helpful to know. The main aim of this review was to exhibit the improvement in power of evaluation and present a new useful method in addition to previous immunoserological ones, for qualitative and quantitative affinity and specificity measurement.

Table 1. A brief comparison between different features of aptamers, antibodies and chemicals.

Attribute	Antibodies	Aptamers	Chemicals	Reference
Size	150 kDa	10–15 kDa	<1 kDa	[8]
Targets	Haptens or macromolecules	Everything including toxic targets from ions to macromolecules	Proteins	[9]
Affinity	nmol–pmol	μmol–pmol	mmol–nmol	[10]
Specificity	Capable to distinguish even a single chemical group difference and conformational differences	Capable to distinguish even a single chemical group difference and conformational differences	Fit to a binding site of a molecule	[11]
Immunogenicity	Yes	No	No	[9,12]
Tissue penetration	Difficulty in penetration	Moderate ability to enter	Enter cells and tissues easily	[13]
Function inside cells	No	Yes	Yes	[14,15]
Availability of antidotes	No	Antisense oligonucleotide	Sometimes	[16,17]
Breaking the interface between macromolecules	Capable to block macromolecules interaction	Capable to block macromolecules interaction	Difficult to break interface between macromolecules	[18,19]
Screening process	In-vivo selection among biological systems	In-vitro combinatory chemistry selection	In-vitro rational design or combinatory chemistry selection	[20,21]
Selection condition	Physiological	Various	Various	[2,22]
Chemical modification	Limited	Various	Various	[23]
Thermostability	Sensitive to temperature	Stable	Stable	[24,25]
Shelf life	~5 years	Unlimited	Unlimited	[26,27]
Stability	Hours to days	Hours to days	Minutes to days	[23]
Nuclease degradation	Not affected	affected	Not affected	[28,29]
In-vitro capability	No	Yes	Yes	[30,31]
Ability to be modified	No	Yes	Yes	[32]
Shipping	Dry ice/overnight	Ambient temperature	Ambient temperature	[33,34]
Detection systems	Well developed but complex	Recently developed but simple	Well developed but complex	[35]
Consistency of performance	Lot-to-lot variation is an issue	Lot-to-lot variation is not an issue	Lot-to-lot variation is not an issue	
Identification	Well developed	Prone to failure	Well developed	[36,37]
Quality control	Well developed	Developing	Well developed	
Cost	Very expensive	Relatively expensive	Inexpensive	[38,39]

Smart aptamers

In a computational process, structural stability and specific binding affinity of aptamers are mathematically predictable using parameters such as $k_{(on)}$, $k_{(off)}$ and k_d, and thermodynamic parameters like in a distinctive biomolecular interaction. These types of aptamers which are selected with predefined equilibrium (k_d), rate (k_{on} and k_{off}) constants and thermodynamic parameters such as ΔH and ΔS are termed 'smart aptamer' and they could be isolated using kinetic capillary electrophoresis [59].

Aptamer characteristics

Structural features of aptamers

Different structures are possible for different kind of RNA, DNA or protein aptamers due to their sequence, physicochemical properties and their target. Three most prevalent predictable forms of aptamers include hairpins (both in RNA and DNA aptamers), pseudoknots (mostly RNA aptamers) and quadruplexes (four-armed structures formed by interaction of four guanine nucleotides) which match together (G-quartet or G_4) [60].

Affinity and specificity

One of the best targets for aptameric selection is proteins. Dissociation constants (K_d) of aptamer-protein complexes usually lie in nanomolar and subnanomolar ranges (10^{-11}–10^{-9} mol/l). K_d is usually 10^{-6} to 10^{-7} mol/l for low-molecular weight targets [61].

Interaction will be more specific if aptamers bind to variable region of a protein molecule, as opposed to the conserved region. This is because of the presence of multiple recognizable epitopes in the variable regions of the proteins [60].

Epitopes recognized by aptamers

As with antibodies, aptamers bind to proteins at specific surface recognition sites called epitopes. Epitopes of proteins have been recognized by most aptamers [60–63]. In contrast to antibodies, native proteins are usually used as targets in aptameric selection. Therefore, it is possible for aptamers to recognize several protein sites in a three-dimensional structure. Compared with antibodies, aptamers are selected within an organism for binding to relatively short fragments of processed protein targets. Site-directed selection of aptamers is a method designed to avoid binding of aptamers to undesirable epitopes or on the contrary, used to recognize a particular epitope of a protein.

SOMAmer arrays

Based on protein-binding and complementary base-pairing features of aptamers, Gold *et al.* [ ] developed a new revolutionary detection and sensing aptamers in the context of proteomics which is called 'slow off-rate modified aptamer' or SOMAmer technology. These are AQ8 the new generation of aptamers. This method extends the possibility of aptamer-based pathogen supervision. The primary innovation in SOMAmer development was stimulated by the idea that aptamers can exhibit protein-like behaviours if we add functional groups to the amino acid side-chains resembling their diverse chemical effects. Therefore, two important innovations in SOMAmers deployed are as follows: having chemically modified nucleotides so they have protein-like properties, and manipulated kinetics to improve the specificity. Incorporation of four modified dUTP analogs into DNA library AQ9 have been done through polymerase extension of a primer annealed to a biotinylated template. The DNA library containing modified nucleotides is incubated with the target molecule after separation from biotinylated templates. The slow off-rate enrichment and partition process is used extensively in washing with large volumes. After separation of the bound DNA from the unbound DNA, the enriched DNA sequences are amplified by PCR to prepare the DNA pool for the next round of selection. Because of the functional groups that mimic amino-acid side-chains, modified DNA library which contain the generated SOMAmers are thought to have some 'protein like' properties. The more different modifications are done on nucleotides by different microbial surface antigens, such as proteins or carbohydrates for SELEX against difficult targets, the more SOMAmers are capable of binding to the target. Massive potential with below 30 nmol pool k_d values has been demonstrated from aptamers successfully obtaining against 1200 human proteins. First, biotinylated SOMAmers are incubated with samples, and then SOMAmer-protein complexes are captured onto streptavidin-coated beads. After washing, modification with a NHS-biotin tag is performed on complex target proteins. Selective disruption of nonspecific binding interactions is done, and the complexes are photo-cleaved from the first set of beads and challenged with a polyanionic competitor (dextran sulphate). The complexes are then attached via the target protein biotin to a second set of avidin-coated beads. Bound SOMAmers are released from their targets and quantified after washing [61].

Aptamers targeting pathogenic determinants

Staphylococcus aureus is associated with nosocomial infections, food poisoning, superantigen-derived diseases (such as toxic shock-like syndrome) and antibiotic resistance. Different virulence factors of *S. aureus* provide AQ10 good targets to determine the presence of pathogen and estimate its pathogenesis. ~~One virulence factor comprises~~

AQ23 **Table 2. Different methods which are used for staphylococcal enterotoxin B detection.**

Methods	Sensitivity	Reference
Biological assays	20 min with a sensitivity of 10–100 pg of SEB/ml of prepared food samples	[67]
Immunological assays		
Gel diffusion assays	0.1 μg/ml	[68]
RPLA	The kits showed high specificity and sensitivity with a detection limit of 0.75 ng enterotoxin/g of food	[69]
Immunoelectrophoresis	Radio-rocket is the most sensitive assay (250 pg/ml)	[65,70,71]
Counter, rocket, fused-rocket and radio-rocket platforms		
ELISA	The sensitivity of this assay reached 0.2 ng of SEB/ml PBS with BSA and fetal bovine serum. It reached 0.39 ng of SEB/ml of 50 g/l skim milk, human urine and water; 1 ng/ml of SEB could be detected in sandwich assay	[72–78]
Indirect, sandwich, competitive		
PCR–ELISA	Sensitivity and specificity of the sec-1 primer set were 100 and 82%, respectively. In a real-time iqPCR method, sensitivity is 1000 times more ~~(<10 pg ml/1)~~ than the ~~in-h30 000 pg/mlouse~~ ELISA and had a dynamic range of approximately 10 pg/ml ~~to approximately~~	[69,79,80] AQ24
ELIFA	The ELIFA system had a turnaround time of approximately 1 h and a detection limit of 1 ng/ml of purified SEB. The ELISA had a total turnaround time of 21 h, or 3 h using plates precoated overnight with the capture antibody. The detection limit of the ELISA for purified SEB was 0.05 ng/ml	[75]
CNT-ELISA	100–100 ng/ml	[81,82]
EMIT	Sensitivity is around 100 ng/ml of samples	[69,74]
POC immunodetection system: ELISA–LOC	POC's sensitivity is 0.1 ng/ml	[83]
FELISA	The described FELISA allows a SEB quantification of 0.1 fg/ml for purified toxin and a detection limit of at least 10 pg/g of contaminated food	[64]
Magnet-beads immunoassay	Magnet-beads immunoassay Sensitivity is 100 pg/ml	[84–86]
Chromatography	The ICT was completed within 30 min, providing a limit of detection close to 20 pg/ml in buffer and showing no cross-reactivity with the other major toxin of the bacterium, staphylococcal enterotoxin A	[84]
TLC, GC, HPLC, column C, ICT		
RIA	1 ng/ml	[87]
Flow cytometry	Sensitivity estimated to be 0.01 and 0.25 ng/ml for buffer and milk samples, respectively	[71]
Molecular probes and PCR	Molecular probes and PCR	[88]

CNT, carbon nanotube; ELIFA, Enzyme Linked Immunofiltration Assay; EMIT, enzyme-multiplied immunoassay technique; FELISA, Fluorogenic Enzyme-Linked Immunosorbent Assay; ICT, immunochromatographic test; iqPCR, immunoquantitative PCR; LOC, Lab-On-a-Chip; RIA, radio-immune assay; POC, point-of-care; RPLA, reverse passive latex agglutination; SEB, staphylococcal enterotoxin B.

AQ11 ~~the staphylococcal enterotoxins of which staphylococcal enterotoxin B (SEB) is one of their most important.~~ Various immunoserological methods were applied in toxin detection.

Staphylococcus aureus and one of its diarrhoea-inducing toxins (staphylococcal enterotoxin B) as a model for detecting and evaluating microbial targets with aptamers

Food-borne diseases are one of the major human health concerns worldwide and bacteria have been the causative agents of two thirds of outbreaks. The general term, poisoning, indicates any type of diseases or illnesses after consuming food products. Among the 50 staphylococcal virulence factors which have been described to date, various enterotoxins are recognized as one of the main causes of gastrointestinal tract poisoning. Staphylococcal enterotoxins are notorious for their possible roles in many other human diseases such as atopic dermatitis, Kawasaki syndrome, nasal polyposis, and certain autoimmune disorders. It is of great importance to develop a quick, specific, sensitive and affordable method for its detection and evaluation.

Common methods of microbial target detection and evaluation

As summarized in Table 2, we can divide routinely used methods for the detection of microbial whole cells and their products into the following 12 main categories: biological assays, immunological assays [64], chromatography assays, radioimmunoassays, flow cytometry, molecular probes and PCR, solid phase surface immunoassay technology, mass spectrometry (MS), matrix-assisted laser desorption/ionization and electrospray ionization, monosaccharide array-based assay, fluorescence resonance energy transfer (FRET), and surface plasmon resonance (does not provide binding efficiency information, but is extremely useful for analyzing biochemical interactions) [65,66]. In 2010, ~~Imani~~ *et al.* [89] reported the antimetastatic effect of intravenous injection of SEB and monophosphoryl lipid A against fibrosarcoma in lung tissue. In addition, in another study, monophosphoryl lipid A (SEB–MPL) treatment led to T-cell stimulation and subsequently cytokine secretion. Results showed that the optimal concentration of SEB and MPL for activation of lymphocytes was 100 ng/μl ($P < 0.05$). Combination of SEB and MPL can cause stronger stimulation and proliferation of mouse lymphocyte cells compared with

Table 3. Purified pathogen molecules and complete bacterial cells and viral particles that have been used in selection of aptamers by systematic evolution of ligands by exponential enrichment.

Groups	Microorganism and/or its product	Complete bacterial cells and viral particles	Reference
Bacteria	*Francisella tularensis* (protein lysate)	*Escherichia coli DH5a*	[94,101,102]
	Campylobacter jejuni (surface extract: protein lysate)	*Staphylococcus aureus*	
	~~*Salmonella enterica*~~, (serovar Typhi type IVB pilus) (outer membrane proteins)	~~*Streptococcus pyogenes*~~	
	Listeria monocytogenes (internalin A)	*Bacillus thuringiensis spores*	
	E. coli (release factor 1) (core RNA polymerase) (lipopolysaccharide O111: B4)	*Strep. pyogenes*	
	Mycobacterium:	*Pseudomonas aeruginosa*	
	M. avium sub. paratuberculosis *MAP0105c* gene product	*M. tuberculosis*	
	M. tuberculosis MPT64 protein	*C. jejuni*	
	M. tuberculosis polyphosphate kinase 2	*Lactobacillus acidophilus*	
	Burkholderia pseudomallei (BipD/BopE/BPSL2748)	~~*Salmonella enterica*~~,	
Viruses	HIV-1 (integrase, reverse transcriptase, nucleocapsid protein, Tat protein, R5 SV, glycoprotein (gp120), drug-resistant reverse transcriptase	Rous Sarcoma Virus particles	[103,104]
	Hepatitis C virus (RdRp, NS3, NS3 helicase, 30X tail, NS3 protease, NS5B RNA polymerase, IRES (internal ribosome entry site)	MS-2 bacteriophage particles	
	Hepatitis B virus (HBsAg)	Mammalian cells expressing hepatitis C E2 envelope glycoprotein	
	Influenza virus (H5N1 HA protein)	Vaccinia-infected mammalian cells	
	SARS coronavirus (NTPase, Helicase)	Human influenza A virus particles	
	Apple stem pitting virus (Coat proteins)	Vaccinia virus particles	
	Foot and mouth disease virus (VP1 protein)		
	Venezuelan equine encephalitis virus (Capsid protein)		
	Ustilago maydis (corn pathogen) (RNA-binding protein Rrm4)		
Parasite	*Leishmania* infantum (H2 antigen)	*Trypanosoma cruzi*	[105,106]
		Live African trypanosomes	
Prion proteins	PrPsc	–	[107]
	PrPsc fibrils		
	rPrPsc		
	rPrPc and mammalian prion proteins		
Bacterial toxins	SEB	–	[38,108,109]
	Cholera toxin		
	Botulinum neurotoxin		
	Shiga toxin		

HBsAg, hepatitis B surface antigen; MS, mass spectrometry; SEB, staphylococcal enterotoxin B.

each component separately [90]. ~~Imani~~, *et al.* also investigated the ~~*invio*~~, induction of necrosis in murine fibrosarcoma via intravenous injection of SEB by ELISA and Flowcytometry [89] and exosome-SEB pancreas, ovarian and breast antitumor activity with electron microscopy and western blotting [91–93].

Selection against purified molecular targets

It is possible to do the selection against different purified targets with fewer SELEX rounds. Numerous proteins and microorganisms such as viruses and bacteria have been used. Structural compounds such as carbohydrates have also been tested as a target. An example is cell-surface extracts of *Campylobacter jejuni*. Two high-affinity DNA aptamers were selected against $MgCl_2$-extracted surface proteins of *C. jejuni* to utilize in magnet bead and red quantum dot-based sandwich assay. Both heat-killed and live bacterial cell were used in this method and final limit of detection report 2.5–250 colony forming unit in buffer and different food matrix [94,95]. Due to *Escherichia coli*, its release factor 1, core RNA polymerase and lipopolysaccharide O111:B4 were targeted for RNA and DNA aptamer selection [94,96,97]. Recently, a DNA aptamer has been designed against VP1 capsid protein of norovirus genotype II-4 [98]. Aspartyl protease is a key protein in morphogenesis of HIV-1 virus. A new RNA aptamer with high affinity against this protein was performed in 2015 [99]. There are key factors in the regulation of protein stability, vRNA, promoter binding and endonuclease activity in connection with amino acid residues in the *N*-terminal of the PA subunit (PA_N) of the influenza A polymerase. Targeting the PA endonuclease domain by an isolated new DNA aptamer led to cross-protection due to influenza A virus infection [100]. Table 3 shows a summary of purified molecules from pathogens against which aptamers have been selected.

Aptamers against live cells

In some cases, aptamers were designed against whole cell of a particular microorganism. In such applications, aptamers may have been restricted ~~use~~ because of the slight differences between the specific binding sites of a natural cell and its purified components. Nevertheless, antiwhole cell aptamers are increasingly becoming

favourites in whole cell SELEX-aptamer (cell-SELEX). One strong reason is that it bypasses complex purification and target-partitioning steps. Bacterial cells present unique challenges over mammalian cells due to their cell-wall structure. Gram-negative bacteria possess a negatively charged outer membrane that can repel nucleic-acid molecules. Capsules covering the surface of many pathogenic bacteria can also obscure target proteins. Both of these factors make it difficult to generate aptamers that bind to the surface molecules of bacterial cells. In addition, bacteria often grow much faster than culture cells of mammalian tissue. Short generation times lead to rapid changes in protein expression and high surface variation between cultures and colonies. Such variation can impede consistent measurement of aptamer binding [110].

In cell-SELEX, pathogenic bacteria are incubated in a solution with a randomized nucleic-acid library. Cell-bound nucleic acids are separated from unbound nucleic acids via centrifugation. Cells are then washed, and cell-bound nucleic acids are eluted using low salt concentrations at high temperature. Cell-bound aptamers can also be amplified directly from the cell surface. Aptamer pools obtained after each round are screened for increased target-cell binding affinity (e.g. by flow cytometric analysis). Rounds of counterselection against nontarget cells, in which the sequences bound to the nontarget cells are removed, can be introduced after the first two rounds of positive selection. Alternatively, positive selection and counterselection rounds can be used [101,102,110–113].

Analytical applications of aptamers

Aptamers are used in a variety of assays that conventionally use antibodies (ELISA-like assays, western blotting, flow cytometry, microscopy, affinity chromatography and capillary electrophoresis). Also, many novel technologies (e.g. aptamer-based biosensors, nanodevices, ligation and amplification assays) are being developed by taking advantage of aptamers. The applications of aptamers derived from whole cell-SELEX are limited, mainly because the number of aptamers made against viral or bacterial targets is limited.

Whole-cell aptamer technologies have potential application against pathogens, or have already been used for pathogen detection: aptamers can be used in sandwich assays such as ELISA antibody-antigen systems, or developed more via flow cytometry techniques instead of using antibodies as a detector probe. In this case, flow cytometry can be used as a useful tool both for evaluating the aptamer target recognition, and for isolating cell populations expressing a target. Briefly, aptamers are fluorescently labelled and used to sort microorganisms. For example, different *S. aureus* strains have been successfully fluorescently labelled by five high-affinity aptamers and differentiated from each other [114]. Flowcytometry was also used for binding affinity determination between target and the aptamer pool obtained from any particular rounds of SELEX. Bacterial cell-SELEX targets against which this technique has been applied include *Lactobacillus acidophilus* [101], *Mycobacterium tuberculosis* [111], *C. jejuni* [112], *S. aureus* [110], *Pseudomonas aeruginosa* [113], *Streptococcus pyogenes* [102]. Due to *L. acidophilus*, six to eight rounds of SELEX was utilized and 164 ± 47 aptamer molecules were bound an apparent K_d of 13 ± 3 nmol [101]. Single aptamer, named 'NK2', has isolated for virulent strain of *M. tuberculosis* (H37Rv) with acceptable affinity. High effects of NK2, proposed its potency as a new antimycobacterial agent [115]. A newer method applied for detection as low as 150 and 760 cells ml^{-1} *S. aureus* in buffer and food matrix (milk) than Aptamer/FITC-based flow cytometry which is called 'aptamer recognition and fluorescent silica nanoparticles' label based dual-colour flow cytometry assay (Aptamer/ fluorescent silica nanoparticles-DCFCM)'. This new method is based on two colour flow cytometry [116].

Recently, flow cytometry has been used to screen aptamers that are specific for vaccinia-virus-infected A549 cells [117]. Aptamers are conjugated to fluorescent nanodye particles increasing fluorescence intensity and detection sensitivity in comparison with the regular flow cytometry labelling during flow cytometry analysis [118].

Using aptamer/antigen interactions for developing biosensors

Diagnostic applications of aptamers primarily focus on the use of aptamers as molecular recognition elements within a larger biosensing system, using the aptamers as probes for target molecules in a similar way to antibody technologies [119]. Almost all biosensors comprise two main parts: a biological molecular recognition element and a signal-transduction element [94,101,114]. One of the best reviews on the role of aptamers for biosensing is written by Torres-Chavolla and Alocilja in 2009 [120]. Aptasensoric detection by electrochemical luminescence sandwich format has been studied [116–118] in which ssDNA aptamers with a high affinity for bacterial spores were conjugated to magnetic beads and used as capture reagents and the biotinylated aptamers were applied as reporters. They have been able to detect even 10 single spores with a linear dynamic range of $10–6 \times 10^6$ [95]. MiladaIkanovi *et al.* [120] showed that aptasensors with fluorescent labels were also used to detect as few as 1000 carbon forming unit (CFU)/ml of *Bacillus thuringiensis* spores to cadmium selenide quantum dots. Using quantum dots conjugation via magnetic beads in a manner of sandwich assay, Bruno *et al.* [121] detected *C. jejuni* by utilizing the bacterial surface protein. This aptasensor could detect both live and heat-killed cells at the range of 2.5 CFU in food complex matrices [95]. In

addition, it is possible to label a polyclonal mixture of aptamers by Alexa Fluor 546–14-dUTP and use in FRET-based assay. Such method was used to detect as few as 25 ng/ml Vp1 structural protein of FMD virus [121]. The same research technology allowed the design aptamers against outer membrane proteins (OMPs) of *E. coli* which were heavily labelled with Black Hole Quencher. This method could detect as few as 30 unlabelled live cells [95,121]. Joshi *et al.* used aptamers to detect ~~*Salmonella typhimurium*~~, OMPs conjugated to magnetic beads without fluorescent labelling which is done through amplifying by PCR. He was successful in detecting 10–100 CFU/ml in normal mode and 4–40 CFU/ml in magnetic-recirculation mode ~~[112]~~.

By using antibody-coated magnetic beads in 2009, Lee *et al.* [122] implied that it is possible to yield a specific aptamer for *E. coli* which is separable within linear dynamic range of $10–10^7$ *E. coli*/ml by the accuracy rate of 10 cells/ml per sample. In another study by Zelada-Guillen *et al.*, two major findings were reported: single-walled carbon nanotube (SWCNT) and DNA capture-element sensing system based on fluorescence quenching. SWCNT is a new label-free rapid method which uses carbon nanotube for specific aptamers by the accuracy rate of 1 CFU/ml in less than 1 min in a linear dynamic range of $0.2–10^3$ CFU. They used SWCNT to create specific aptamer for ~~*Salmonella typhi*~~ type IV pili and DNA capture-element for the sensing of *Bacillus thuringiensis*, *B. anthracis* spores, botulinum neurotoxin and MS-2 bacteriophage [120]. Ohk *et al.* [110] invented an optic-fibre aptasensor with fluorescent labelling which detected *Listeria monocytogenes*; this aptasensor could detect as few as 10^3 CFU/ml of bacteria, and was able to detect contaminated poultry and meat products such as turkey, chicken and beef. Lee *et al.* [123] succeeded in producing a portable sensor platform chip to detect food contamination with toxins and disease-related metabolites by assembling carbon nanotubes on SiO_2 which was laid down with gold electrode with immobilized aptamer on it. This is an aptamer sandwich-based carbon nanotube sensor strategy which is capable of differentiating between similar molecular species with single-carbon-atomic resolution. To enhance the specificity, the procedure can be modified using biotin resulting in sensitivity as low as 10 fmol [123]. Maurer *et al.* [81] created another nano-featured biosensor platform for sensitive, selective and rapid detection of *E. coli* on the basis of carbon nanotubes embroidered with gold nanoparticles modified with a specific, surface adherent RNA sequence.

Cell labelling and imaging

Aptamer-fluorescent conjugate could be considered as a useful bioimaging tool in association with flow cytometry [124]. *Trypanosoma cruzi* specific aptamers were used as probes for the parasite's surface antigens [125] labelling. Fluorescence in situ hybridization has been applied to label specific DNA aptamer against *P. aeruginosa* [126]. Optical microscopy has also been used to visualize the density of aptamer-captured cells on a microfluidic device [127]. Although it is easier for aptamers to label molecules on the cell surface, they have also been used for intracellular imaging. Imaging of bacterial RNA in live cells (*E. coli*) has been accomplished using protein complementation regulated by the interaction of an RNA-binding protein and an aptamer. The RNA-binding protein is engineered to express GFP only in the aptamer-bound state; mRNA and rRNA can be tagged with aptamer and be localized [128] (Table 3).

Therapeutic potential of aptamers

Several therapeutic applications have been defined for the aptamers including new drug design, drug delivery and tissue targeting [129]. For example, in the field of new drug design, Pfizer and Eyetech Pharmaceuticals developed a pegylated-single strand aptamer with specificity to vascular endothelial growth factor (VEGF 165). VEGF 165 plays an important role in membrane permeability and angiogenesis [129]. Novel combination therapies with an anti-VEGF and antiplatelet-derived growth factor (anti-PDGF) could be the next big step ahead for the treatment of wet-age-related macular degeneration. Fovista is a new anti-PDGF phase-IIb aptameric drug compatible with ranibizumab. A randomized controlled phase II-b study assessed efficacy and safety of Fovista and showed that 0.3 mg in combination with Lucentis (the commonly prescribed drug for preventing age-related macular degeneration treatment) is very effective. A new phase II clinical trial aptamer drug REG1 has been developed by Regado Bioscience as anticoagulation drug. REG1consists of two components: RB006 (coagulation factor IXa-specific aptamer) and RB007 (oligonucleotide antidote of the RB006 aptamer) protecting the aptamer against nuclease-mediated degradation, RB006 (a 2′-ribo purine/2′-fluoro pyrimidine aptamer) conjugated to a 40 kDa PEG tail.

There are many aptamer-based drugs which are currently in clinical trials such as NU172 (a thrombin-specific aptamer) for anticoagulation, leukaemia, ARC1779 (a von Willebrand factor-specific aptamer) for carotid artery disease and AS1411 (a nuclein-specific aptamer) for acute myeloid leukaemia [28,129,130]. Aptamer-based products are developed through different channels. Cell-SELEX creates aptamers which can be used in reverse chromatographic strategies for tumour cell marker purification and identification.

Aptamers can even recognize molecular differences between patient's specific cancer markers in T-cell acute lymphoblastic leukaemia [131]. In-silico data have been incorporated to reduce the number of SELEX round. To achieve this, new genetic algorithm has been designed for post-SELEX screening. This method decreased the

number of SELEX rounds to three to predict an antiprostate antigen aptamer [132].

S. aureus is a pathogen with various potent toxins including the enterotoxins. Spiegelmer is a mirror-image aptamer for SEB designed in 2003 [133] which was followed by preparation of an ssDNA aptamer against SEB that was isolated by the magnet-bead method in 2012 [38]. Aptamers to other enterotoxins were introduced for staphylococcal enterotoxin A in 2014 [134] and for staphylococcal enterotoxin C1 in 2015 [135]. Microbial toxins always have been good candidates to isolate aptamers. Specific RNA aptamer with protective effect on ricin target its A-chain structure [136]. As aptamers have the potential to boost the human immune system, they can be used as antimicrobial products. 'NK2' aptamer is an anti-tuberculosis drug with high affinity to *M. tuberculosis* (H37Rv) virulent strain which has been introduced by Chen *et al.* [100] with no cross-resistance with other known antimycobacterial agents. Aptamers amplify the secretion of interferon-γ in human body via a $CD4^+$ T-cell mechanism.

In another study, OMPs of *Salmonella enterica* serovar typhimurium have been targeted for aptamer isolation. In this study, a new sensitive DNA aptamer was presented by using gel-shift analysis. Counterselection of *E. coli* LPS and OMPs was chosen to improve their aptamer accurate detection power and evaluation. MS and southern/western blot analysis was used for OMPs identification [137]. A list of aptamers which have progressed to clinical trials was published by Stoltenburg *et al.* [2] and Bunka *et al.* [12] (Table 3). Aptamers may undergo conformation changes during binding into biotoxins so it is possible to monitor changes in the distance between two fluorophores (a donor and an acceptor). This phenomenon also happens during FRET [72]. SOMAmer arrays have been successfully applied to identify novel biomarkers in chronic kidney disease and nonsmall-cell lung cancer. SOMAmer and array technology were potentially developed for pathogenic species and strain typing, pathogen surveillance, and detection of different microbial infections. They can differentiate between the proteome profiles of pathogens and healthy humans. Another usage of SOMAmers is comparison of antibiotic resistance profiles of different bacterial species/strains, comparison of proteome profiles or multiplex antimicrobial resistance screening among patients [138]. SEB is a major virulence factor in toxic shock syndrome and staphylococcal food poisoning. A DNA aptamer with $k_d = 64$ nmol was isolated with neutralization properties against SEB [139].

Drug delivery

Another useful application of aptamers is to apply them as a drug delivery vehicle into the target cells. Prostate-specific membrane antigen (PMSA) has been targeted which is an important prostate cancer marker in patients [140]. There are two aptamer designs for two kinds of cell lines: DUP-1 aptamer for negative prostate-specific membrane antigen prostate cancer cells and aptamer A10 for positive cancer cells. A dual anticancer drug-aptamer constructs which is a combination of doxorubicin and A10 was introduced to the prostate cancer cells and it acted effectively. As a new therapeutic agent, small interfering RNAs (siRNAs) have been considered for prostate cancer therapy. siRNAs have potential to interfere with a series of gene expression regulation utilizing RNA interference pathway. For effective treatment via siRNAs, safe mode of delivering is important. Chu *et al.* [141] introduced an effective anti-PSMA aptamer-siRNA conjugate with a streptavidin bridge for prostate cancer cells (LNCaP). Also, aptamers could be designed against cell membrane receptors and be utilized in clinical investigations [142]. They could improve the delivery of cancer drugs and carry their specific loaded active drug to the target cell for therapeutic purposes. When used as nanoparticles, aptamers are a good choice to combat against molecular mechanisms of drug resistance [143]. Surpassing other chemical and molecular probes such as monoclonal antibodies, aptamer-mediated therapy has matured in the field of DNA hydrogels, carbon nanotubes and other nanomaterials [144]. Zhang *et al.* [145] presented a new aptamer-encapsulation system because of the expansion of new antimicrobial drug delivery systems. Chimeric aptamer conjugated with locked nucleic acids facilitated the accuracy of drug delivery and targeting in the preclinical trials [146].

AQ19

Application via bioimaging

Aptamers bound to a quantum dot, fluorophore or other materials such as gadolinium (used for MRI measurements) are used in bioimaging. The advantage of aptamers for bioimaging is their safety and nontoxicity for human body because of the presence of oligonucleotide moieties in body. Aptamers with their high specificity for their target, rapid diffusion through the blood stream circulation and accurate targeting can enhance the results during clinical analysis or diagnosis.

Based on these advantages, aptamers have been studied as imaging agents for cell imaging as well as single-protein imaging. C6 cell imaging has been achieved using a Cy3-labeled AS1411 aptamer which included a chemical modification of 5-(N-benzylcarboxyamide)-2′-deoxyuridine (called 5′-BzdU) on a thymidine base [147]. AS1411 aptamer is a specific aptamer which has been designed for nucleolin transmembrane protein in cancer cells. In AS1411, binding affinity to specific target is improved by chemical modification. Cell bioimaging is chemically

modified by Cy3-labeled AS1411 aptamer was more efficient for C3 cells than the original Cy3-labelled AS1411 aptamer. There are other aptamer complex such as QD-1 and DUP-1 which are specific for PSMA (+) and PSMA (−) prostate cancer cells (LNCaP and PC3). It has been shown by bioimaging that these aptamers just bind to prostate cancer cells and not to other cancer cells or normal prostate cells (PNT2) [148]. In addition, there are aptamers directed against p68 liver tumours and specific small cell lung cancer cells that also had the potential for use as bioimaging probes [72,149,150]. Based on excellent optical properties of carbon nanodots (e.g. emission), Lee *et al.* [151] decorated a new aptamer-conjugate probe for bioimaging in cancers. Guanine quadruplex is one type of structural DNA aptamer. A DNA Guanine quadruplex aptamer has been used as a stabilizer of fluorescent silver nanocluster in cell bioimaging [152].

Western blot analysis

There are a lot of companies which produce several antibodies against different proteins. A new western blot strategy which uses an aptamer to detect the target protein has been introduced making it a one-stop technique. There is a His-tag specific quantum dots conjugate RNA aptamer which was used in their product instead of using two types primary and secondary of antibodies. The most valuable benefits of their products are that they are less time-consuming, and do not require P^{32} and are feasible in multiplex detection [153]. In another study, a DNA aptamer isolated under magnetic separation method for thyroid transcription factor 1 was found useful when applied in ELISA and western blot affinity purification [154]. A series of aptamers have also been developed to detect diverse plant virus which can be utilized in dot blot and western blot analysis [155].

AQ20

Aptamer affinity chromatography

Immunoaffinity purification is used to purify target proteins. This technique relies on the interaction between an antigen and an antibody. This is a common laboratory technique in most scientific fields. Equal or superior affinity and specificity to the target, a smaller size, higher productivity, and better stability are all advantages of using an aptamer in chromatography.

The small size of aptamers and their ease of use as a conjugative-chromatographic support make them good candidates for affinity separation in affinity chromatography [156]. For example, a high-performance affinity chromatography which is based on DNA aptamer against lysozyme was developed and introduced in 2012 [157]. Romig *et al.* [158] developed an aptamer affinity chromatography system for human L-selectin. The recombinant human L-selectin-immunoglobulin fusion protein was successfully purified from Chinese hamster ovary cell-conditioned medium using this aptamer affinity column [158]. Additionally, it was demonstrated that sandwich aptamer affinity chromatography using two aptamers improved the sensitivity and selectivity for thrombin [156]. Another achievement in aptamer use in affinity chromatography refers to microbead-based affinity chromatography chip (μ-BACC) which was developed for the separation of hepatitis C virus (HCV) RNA polymerase protein by using a mixture of RNA aptamer-immobilized beads [159].

Conclusion

Aptamers can be developed against chemical and biological molecules including toxins which are a feasible and economical approach. Aptamer isolation bypasses the in-vivo route; therefore, it is much easier to work with aptamers compared with the commercially available monoclonal antibodies.

Modified aptamers can be applied to various targets especially those molecules with detection limitations with antibodies. Aptamers can penetrate biological membranes and the sites of immune systems that cannot be reached by conventional techniques to interact with specific target molecules. One of their best advantages over antibodies is once one discloses the aptamer's sequence, other scientists will be able to synthesize and use it in their own research. Altogether, aptamers have the potential to be used as substitutes for traditional immunoserological methods to detect targets such as microbial toxins in food matrices, human tissues, and serum samples. They can be introduced as a new generation of therapeutic drugs as well as alternatives for traditional detection kits. Aptamers also have potential to use as a drug delivery vehicle or an engineered construct in biotechnology.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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