Commentary

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Bioanalysis

Aptamers in diagnosis and therapeutics against the antimicrobial-resistant microorganisms: recent trends and challenges

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The emergence of multidrug-resistant strains

Genetic mutations and indiscriminate use of antimicrobials, particularly in developing countries, have given rise to widespread antimicrobial resistance. Falling efficacy of the available antimicrobials, such as the β-lactams, aminoglycosides, fluoroquinolones, macrolides, and tetracyclines against many bacteria including the pathogenic ones, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Mycobacterium tuberculosis*, *Neisseria gonorroheae,* or viruses, such as hepatitis B, hepatitis C, influenza, and fungi, such as *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus* poses a significant challenge for the healthcare sector [1]. Apart from just the challenge of treating such resistant strains, a significant hurdle exists at the front of detecting these pathogens, especially when part of a larger conglomerate, such as in the biofilms. Moreover, the incidence of multidrug-resistant strains is increasingly noticed in hospital setups, that is, nosocomial infections; and are noted for simultaneous resistance against multiple (broad-spectrum) antimicrobials. Unfortunately, the current microbial detection platforms like the microbiological isolation, polymerase chain reaction-based or immunologic techniques, especially in aqueous samples – a significant resource for the resistant strains – suffer from lack of sensitivity and cost-effectiveness, are cumbersome, and often inadequate for point-of-care diagnostics.

The aptamers

Aptamers, usually a single-stranded DNA or RNA fragment folded within a 3D space, exhibit affinity toward various target molecules enabling molecular recognition with high resolution, for example, between enantiomers, protein isoforms or molecules differing by mere a functional unit [2]. Over the last few decades, aptamers have caught the attention of microbiologists as a useful tool for detecting microorganisms with excellent stability and precise targeting – both traits often missing in antibodies. With advanced synthetic techniques, the aptamers can now be edited at a molecular level, further encouraging their uses as recognition/reporter molecules for a multitude of pathogens. Moreover, aptamers can be tailor-made against almost any molecule, including the nonimmunogenic ones; further expanding the landscape of target analytes it can bind and facilitate detection.

Synthesis of aptamers: SELEX

Aptamers are produced from a pooled library of oligonucleotides with random regions (20–60 nt) through a method comprised of a sequence of steps, as mentioned below, and known as the Systemic Evolution of Ligands by Exponential (SELEX) technique:

- Selection: the library of oligonucleotides, with or without labeled with fluorophores or radiolabels, and with an affinity toward the target molecule may contain up to 10^{16} distinct sequences, where an unknown/random

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region – chosen based on the size of the target molecule, diversity and cost – is sandwiched between two flanked sequences.
- Partitioning: the sequences bound to the target molecule are separated/partitioned from the unbound fractions by mass-separation techniques, such as size-exclusion or washing.
- Amplification: multiple copies of the sequences are prepared by polymerase chain reaction to enrich the library for further applications. In contrast, the flanked sequences are used as primer-binding sites.

Usually, 5–15 cycles of SELEX are reiterated to prepare aptamers with a high affinity toward the target analyte. Both DNA and RNA libraries are used: DNA aptamers are comparatively less susceptible to cellular nucleases than the RNA ones, although less stable biologically. RNA aptamers, on the other hand, can form more secondary structures, albeit more immunogenic and vulnerable to nucleases – thus, often require chemical modifications.

Antibacterial aptamers

- *Salmonella:* aptasensors developed by conjugation of aptamers to nanoparticles (NPs), nanotubes (NTs) or chemical groups were reported. For example, biotin-conjugated DNA aptamers S8-46 (K_d = 0.74 μM) and S8-7 (K_d = 1.73 μM) achieved detection limits of 3.5×10^2–3.5×10^3 colony-forming unit (CFU)/ml in *S. typhimurium* suspensions. A system consisting of a mixture of aptamer-coated magnetic iron oxide (Fe_3O_4) NPs and fluorescent ($\lambda_{ex/em}$ = 327/612 nm) cadmium telluride (CdTe) quantum dots bioconjugated to single-stranded DNA (ssDNA) was able to detect *S. typhimurium* with a detection limit of single CFU/ml in food matrices [3]. Similarly, a DNA aptamer-conjugated gold nanodimer consisting of gold nanoparticles (AuNPs) of 35 nm and 15 nm sizes was used to detect *S. typhimurium* (detection limit of 35 CFU/ml) with the help of surface-enhanced Raman spectroscopy [4].
- *Staphylococcus:* a range of aptamers (RAB10, RAB20, RAB28 and RAB35) developed against the whole cell of the *S. aureus* with a K_d of 34–128 nM and detection limit of 100 CFU/ml was successfully used to detect the pathogenic bacteria in a dual-labeled sandwich detection system [5]. A G-quadruplex aptamer (PA#2/8) raised against the Protein A of *S. aureus* could successfully detect the pathogen in an enzyme-linked oligonucleotide assay setup with the highest affinity recorded as $K_d = 11.3 \pm 1.4$ nM [6]. Similarly, ssDNA aptamers were used successfully to detect *Staphylococcus epidermidis* in platelet concentrates [7].
- *E. coli:* a quartz crystal microbalance-based ssDNA aptasensor was developed with 19 iterations of SELEX targeting the whole cell of *E. coli* O157: H7 followed by six rounds of counter-selection against a range of pathogens, such as the *S. aureus*, *S. typhi* and *Listeria monocytogenes* [8]. The aptasensor showed excellent efficiency in detecting the pathogen (detection limit of 1.46×10^3 CFU/ml) within 50 min.
- *M. tuberculosis:* a ssDNA aptamer targeting the MPT64 protein secreted by the microbe was used for electrochemical impedance spectroscopy and was able to detect the bacterium in sputum samples from TB patients with a detection limit as low as 4.1 fM [9]. Similarly, slow off-rate modified aptamer based reagents developed against a series of antigens (85A, 85B, 85C, GroES, GroEL2, DnaK, CFP10, KAD, CFP2, RplL, Tpx) enabled detection at sub-pM levels of the bacteria in 40% of the assayed serum samples [10].

Antiviral aptamers

- Hepatitis: a fluorescent DNA aptamer developed against the hepatitis B envelope-antigen was able to detect the hepatitis B envelope-antigen in patient serum within 2 min with a detection limit of 609 ng/ml [11]. Similarly, a chemiluminescent aptasensor using ssDNA aptamers and targeting the hepatitis B surface antigen was able to detect the antigen (immobilized on carboxylated magnetic nanoparticles) in serum samples with a detection limit of 0.1 ng/ml [12]. In a recent study, atomic force microscopy tips functionalized with aptamers targeting the core antigen of hepatitis C virus was able to detect the antigen in serum samples from patients [13].
- HIV: an RNA aptamer targeted against the HIV-1 aspartyl protease enzyme, which helps in the morphogenesis and replication of the virion, showed excellent affinity to the enzyme *in vitro* (K_d = 2–22 nM) and inhibition of the enzyme activity [14]. In a separate study, aptamers developed while targeting the 2′-fluoroarabinonucleic acid (FANA) showed quite a high affinity (K_d = 50–100 pM) *in vitro* toward the HIV-1 integrase enzyme known to induce the incorporation of the viral DNA into the host genome [15].
- Influenza: a range of ssDNA aptamers targeting the immunogenic HA1 protein of the subtype H1 influenza virus showed excellent binding to the antigen ($K_d = 64.76 \pm 18.24$ nM, 69.06 ± 12.34 nM and 50.32 ± 14.07 nM for the three prototypes) *in vitro* [16]. Interestingly, these aptamers showed comparatively much lesser binding

to the HA1 obtained from the H5 subtype of the virus – thus demonstrating excellent sensitivity. In another study, a series of aptamers targeting the N-terminal of the PA subunit of the influenza virus showed an adequate binding affinity toward the PA antigen. One of the aptamers even provided cross-protection against the infections of H1N1, H5N1, H7N7 and H7N9 viruses with a half-maximal inhibitory concentration (IC_{50}) of approximately 10 nM.

Antiparasitic aptamers

- *Trypanosoma brucei:* RNA aptamers were developed targeting the 24 kDa protein at the flagellar pocket of the unicellular parasite – the causative agent of sleeping sickness – and showed adequate binding to the parasite (K_d = 60 nM) while in the bloodstream [17]. The aptamer was readily internalized by the parasite and can be used for drug delivery. A 2′-fluoro analog of the RNA aptamers (cl57), targeting the VSG surface protein of the parasite and bioconjugated to single-walled carbon nanotubes (CNTs), was developed into a potentiometric sensing platform capable of detecting the parasite in blood samples from patients [18].
- *Trypanosoma cruzi:* a modified 2′-fluoro RNA aptamer developed against the pathogen, known to cause the Chagas disease, were able to demonstrate a 50–80% inhibition of the parasitic invasion of LLC-MK_2 monkey kidney cells at 1 μM concentration [19], while also showed potential for detection of the parasite in the serum of infected mice.

Aptamers against the formation of biofilms

Formation of biofilms on surfaces, as noted in approximately 65% of all the infections, is a significant mechanism of antimicrobial resistance. The polysaccharide-rich biofilms act as an impermeable barrier to the antimicrobial molecules and, thus, the alleviated efficacy of the antimicrobial agents. A series of ssDNA aptamers [20] targeting the flagellin protein of the *Salmonella choleraesuis* bacterium showed excellent affinity (K_d = 41 ± 2 nM). By inhibiting the flagellin protein, these aptamers were able to inhibit the formation of biofilm by the pathogen and made the microbe more vulnerable to the antimicrobials, such as ampicillin. In another study, a family of three DNA aptamers, Lyd-1, Lyd-2, and Lyd-3, targeting *Streptococcus pneumoniae*, were synthesized and later integrated into graphene oxide (GO)-based fluorometric assay [21]. The three aptamers showed considerable affinity toward the microbe with the K_d values of 844.7 ± 123.6, 1984.8 ± 347.5 and 661.8 ± 111.3 nM for the Lyd-1, Lyd-2 and Lyd-3 aptamers, respectively.

Challenges in translation

Despite the advantages offered by the aptamers over antibodies, its success in translation to yield real therapeutics has been less than spectacular due to the following reasons:

- Biodegradation: Upon administration, the aptamers are susceptible to both the endonuclease and exonuclease enzymes that cleave the molecules and mask its therapeutic effect. Chemical modification of the nucleotide molecules or the phosphodiester linkages has been noticed to deter any such biodegradation, although the success so far has been limited. The use of xeno nucleic acids – synthetic nucleic acid analogs that mimic the DNA or RNA, such as the threose or peptide nucleic acid – can also be an option to replace the aptamers as a more stable therapeutic agent with longer circulation half-life ($t_{1/2}$), albeit with comparable efficacy [15].
- Renal clearance: aptamers, due to their molecular weights often below the threshold for renal clearance (30–50 kDa), are rapidly cleared from the bloodstream by renal filtration. On the contrary, antibodies, as much bulkier molecules (≥150 kDa), exhibit longer circulation times in the bloodstream. Bioconjugation of the aptamers with polymers, such as polyethylene glycol (PEG) of high molecular weight, confer extended circulation times to the aptamers apart from improved stability and alleviation of toxicity.
- Toxicity: the toxic reactions of the aptamers are immunogenic reactions producing antibodies noted particularly after repeated administration and polyanionic effects, including binding to the proteins present in blood serum – thus, causing unwanted sequestration in organs and curtailment of its therapeutic effects. Chemical modification of the aptamers, such as PEGylation, is known to trigger the production of antibodies against the synthetic ligands. Moreover, molecules such as PEG are known to cause allergic reactions after administration due to the pre-existing antibodies.

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Future perspective

At this stage, there is hardly any detailed information on the prevalence of antimicrobial resistance within the commonly encountered microorganisms. Unfortunately, the situation is not improving and needs urgent attention on a global scale to avoid a catastrophe in public health. Microorganisms rely on multiple mechanisms, from mutated genetic makeup to the formation of biofilms, to exhibit such resistance. Often, the causative organisms present a mix of both sensitive and resistant strains with considerable phenotypic and genotypic overlap making it difficult to segregate the resistant strains from the sensitive ones. Moreover, due to a plethora of reasons, including regulatory obstacles and limited profit margins faced by the pharmaceutical companies while developing novel antimicrobial molecules, only a handful of the antimicrobial formulations are currently undergoing Phase II or III trials.

Aptamers are providing novel avenues for the detection and analysis of microbial organisms in real-life pathological samples, such as blood, urine and sputum. So far, antibodies have remained the mainstay of the current paradigm in such biosensing platforms. However, aptamers present multiple advantages over the conventional antibody-based platforms on the merits of being reusable, stable and most importantly, its precise targeting capability and high affinity toward a range of molecules including small molecules like a toxin, antibiotics, peptides, genetic materials; or even the entire cells. Moreover, aptamers can be generated against multiple targets including the various components of the microbial cells (for example, cell walls, pili, secreted proteins) – a feat difficult to achieve with antibodies due to a gamut of issues including lack of sensitivity, cross-reactions, inflated cost and time constraints. Such high specificity of the aptamers may be used to distinguish the resistant strains from the sensitive strains based on granular structural or genetic details, which has remained an elusive goal with the antibodies. The performance of such aptameric sensors while dealing with real-life pathological samples has overall been satisfactory so far. However, both the sensitivity and specificity of such sensors are yet to stand the rigor of large-scale trials.

In a nutshell, aptamers are slowly getting established in the field of biosensor development, and more translation of such aptamer-based sensing platforms into real products can be envisioned for the future. However, the existing challenges in the translation of aptamer technology, including its vulnerability toward enzymatic degradation and rapid renal clearance, need to be addressed. The integration of nanotechnology with aptameric constructs provide fresh hopes and is worth exploring. Nanomaterials, due to their small sizes (often <100 nm), exhibit unique materialistic properties, including electrical and photochemical attributes. Thus, emerging nanomaterials, such as graphene, quantum dots, CNTs and AuNPs, present exciting opportunities in designing highly sensitive electrochemical as well as optical sensors based on surface plasmon resonance and surface-enhanced Raman spectroscopy. Such proposed convergence of nanotechnology with aptamers is also rational, given the high surface area of the nanomaterials that provides an adequate area for grafting the aptamers.

Additionally, with advanced synthesis and growing knowledge in nanochemistry, control over conducting such bioconjugation has improved drastically over the last decade. Incorporation of microfluidic technology into the design of such aptameric biosensors, although sparsely reported, is a welcome development and should be able to offer a range of added benefits, including miniaturization, integration, automation and high-throughput screening. The prospects are further elevated due to the improvement of the SELEX technique, particularly in separating the target-bound nucleotides from unbound bits, which remains a crucial requirement in improving the performance of aptameric biosensors. Introduction of techniques, such as capillary electrophoresis, atomic force microscopy, surface plasmon resonance and flow cytometry in tandem to the conventional methods of separation, such as centrifugation, filtration and chromatography, is further guiding toward effective separation protocols in addition to knowledge regarding affinity between the targets and aptamers.

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