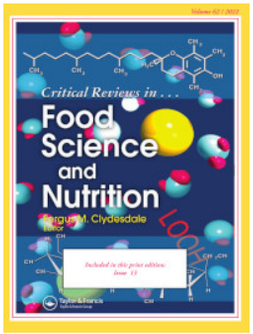

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Critical Reviews in Food Science and Nutrition

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/bfsn20

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To cite this article: Hua Ye, Zhixin Yang, Imran Mahmood Khan, Sobia Niazi, Yuanxin Guo, Zhouping Wang & Hongshun Yang (2022): Split aptamer acquisition mechanisms and current application in antibiotics detection: a short review, Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2022.2064810

To link to this article: https://doi.org/10.1080/10408398.2022.2064810

Published online: 04 May 2022.

CRITICAL REVIEWS IN FOOD SCIENCE AND NUTRITION
https://doi.org/10.1080/10408398.2022.2064810

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REVIEW

Split aptamer acquisition mechanisms and current application in antibiotics detection: a short review

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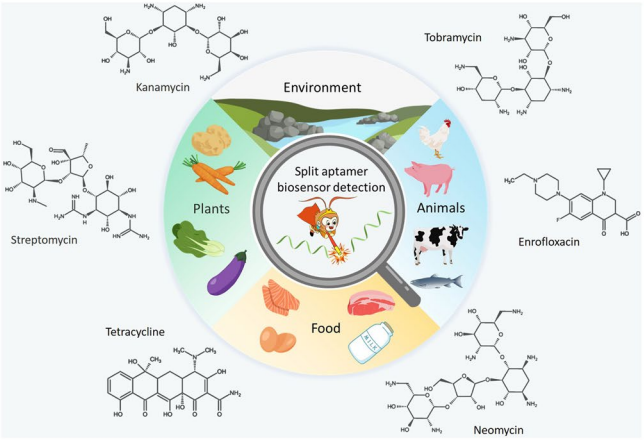
ABSTRACT

Antibiotic contamination is becoming a prominent global issue. Therefore, sensitive, specific and simple technology is desirable the demand for antibiotics detection. Biosensors based on split aptamer has gradually attracted extensive attention for antibiotic detection due to its higher sensitivity, lower cost, false positive/negative avoidance and flexibility in sensor design. Although many of the reported split aptamers are antibiotics aptamers, the acquisition and mechanism of splitting is still unknow. In this review, six reported split aptamers in antibiotics are outlined, including Enrofloxacin, Kanamycin, Tetracycline, Tobramycin, Neomycin, Streptomycin, which have contributed to promote interest, awareness and thoughts into this emerging research field. The study introduced the pros and cons of split aptamers, summarized the assembly principle of split aptamer and discussed the intermolecular binding of antibiotic-aptamer complexes. In addition, the recent application of split aptamers in antibiotic detection are introduced. Split aptamers have a promising future in the design and development of biosensors for antibiotic detection in food and other field. The development of the antibiotic split aptamer meets many challenges including mechanism discovery, stability improvement and new biosensor development. It is believed that split aptamer could be a powerful molecular probe and plays an important role in aptamer biosensor.

KEYWORDS

Antibiotics; biosensor; detection; mechanisms; split aptamer

GRAPHIC ABSTRACT



HIGHLIGHTS

- Currently reported antibiotic-targeted split aptamers are summarized.
- The acquisition mechanisms of antibiotic-targeted split aptamer are discussed.
- Recent application in detection of antibiotics based on split aptasensors are reviewed.
- Challenges and future perspectives of split aptamer-based antibiotics biosensors are discussed.

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#These authors contributed equally to this work.

Introduction

Aptamers are usually obtained through systematic evolution of ligands by exponential enrichment (SELEX) technology from artificially synthesized library through repeated screening steps such as incubation binding, elution separation, PCR amplification, single-strand preparation, etc. (Tuerk and Gold 1990). They are essentially single-stranded DNA (ssDNA) or RNA molecules with approximately 8–100 bases, molecular weight of 2.5–32 kDa, and size of around 1–2 nm (Macedo and Cordeiro 2017). Aptamers can form three-dimensional structures such as stem-loops, hairpins, G-quadruplexes, pseudoknots and bulge structures and bind specifically to certain targets (such as bacteria, toxins, proteins, metal ions, etc.) (Chen, Yan, and Yang 2016a; Kwon, Ahmad Raston, and Gu 2014; Schmitz et al. 2020; Zhang et al. 2016). Given its highly specific recognition of target, aptamers are also hailed as artificial antibodies (Nur Topkaya and Cetin 2021). Inspired by antibody techniques, an increasing number of split aptamers were reported, and various split-aptasensors with high sensitivity and specificity were constructed in the past decades (Qi et al. 2020). According to Chen's review published in 2016, only 6 small-molecule-binding aptamers have been successfully constructed into split aptamers, pointing out the difficulty of aptamers split engineering (Chen, Yan, and Yang 2016a). After 5 years, the number of reported split aptamers increased to 16 kinds, including 5 antibiotic-targeted ones (Qi et al. 2020). However, there is lack of review on antibiotic-related split aptamer and their acquisition mechanism is still unclear. Based on this, in this review, the acquisition mechanisms and application of split aptamer in antibiotic analysis were discussed and summarized, and the future development of split aptamer was prospected, with hope to provide theoretical guidance for the development of aptamer split engineering and the development of sensitive detection methods for antibiotics.

Antibiotics pollution in the environment and their detection techniques

Antibiotics circulating in the environment

Antibiotics are a class of compounds that can kill or inhibit the growth of microorganisms (including bacteria and fungi) (Yang et al. 2018). Currently, they can be divided into two categories: natural antibiotics and synthetic antibiotics (Ribeiro Da Cunha et al. 2019). Natural antibiotics are mainly produced by bacteria and fungi (Grenni et al. 2018). The majority of clinically used antibiotics are natural products extracted from microbial culture, chemically synthesized or semi-synthesized natural product derivatives (Walsh and Fischbach, 2009). According to their chemical structure, antibiotics can be classified into different groups such as β-lactams (e.g., penicillin, cephalosporins) (Bush and Bradford 2016), aminoglycosides (e.g., tobramycin, kanamycin) (Krause et al. 2016), macrolides (e.g., erythromycin, azithromycin) (Chen and Zhou 2014; Sabri et al. 2020), quinolones (e.g., ciprofloxacin, enrofloxacin) (Li et al. 2011; Pham, Ziora, and Blaskovich 2019), tetracyclines (e.g., oxytetracycline and chlortetracycline) (Li et al. 2011; Daghrir and Drogui 2013), sulfonamides (e.g., sulfadimidine) (Nan et al. 2017), etc.

Due to their therapeutic effect on pathogen-induced infections, antibiotics are widely used in modern healthcare and livestock and poultry feeding industry (Chen, Ying, and Deng 2019). However, misuse and abuse of antibiotics on livestock and poultry can cause serious antibiotic residues in animal tissues (Bacanlı and Başaran 2019; Baquero, Martínez, and Cantón 2008; Chen, Ying, and Deng 2019; Gothwal and Shashidhar 2015; Xie, Shen, and Zhao 2018). These kinds of foods with excessive levels of antibiotics may affect the normal microbial flora in the human body (Francino 2015), resulting in allergic reactions (Blumenthal et al. 2019), impaired liver function (Hoofnagle and Bjornsson 2019) and other adverse reactions, thus a potential threat to human health (Ben et al. 2019). Furthermore, wastes from humans and animals containing antibiotics, including urine, feces and wastewater from farming facilities, etc. are constantly released into and accumulated in the environment. And antibiotics in the environment can be taken up by vegetables, crops, aquatic plants, etc. This results in antibiotics circulating through environment, plants, animals, food and human body (Figure 1) (Hu, Gao, and Zhu 2017). Therefore, the sensitive detection of antibiotics is of great significance to ensure food safety and human health.

Traditional detection techniques for antibiotics

Traditional antibiotic detection methods can be classified into microbial detection, physicochemical detection and immunological analysis method (Nan et al. 2017). The microbial detection method refers to the qualitative and quantitative analysis of the inhibitory effect of the antibiotic on the metabolism and physiological function of specific microorganisms, like the TTC-test (2,3,5-Triphenyte-trazoliumchloride), which was widely used in early times (Sabaeifard et al. 2014). Despite the low cost, the microbial detection is time consuming and difficult to control. Commonly used physicochemical detection methods like high-performance liquid chromatography (HPLC) (Wei et al. 2011), gas chromatography (GC) (Chiavarino et al. 1998), liquid chromatography-tandem mass spectrometry (LC-MS) (Fedorova et al. 2014) and gas chromatography-mass spectrometry (GC-MS) (Velicu and Suri 2009), etc. are highly sensitive, but these methods are time-consuming and require complicated pretreatment, expensive equipment, and experienced personnel to operate (Wu et al. 2015). Immunological analysis is a highly selective biochemical method based on specific antigen-antibody interaction, which has been widely reported in antibiotic detection. Ahmed et al. (2020) made a comprehensive summary of the immunodetection methods of antibiotics,

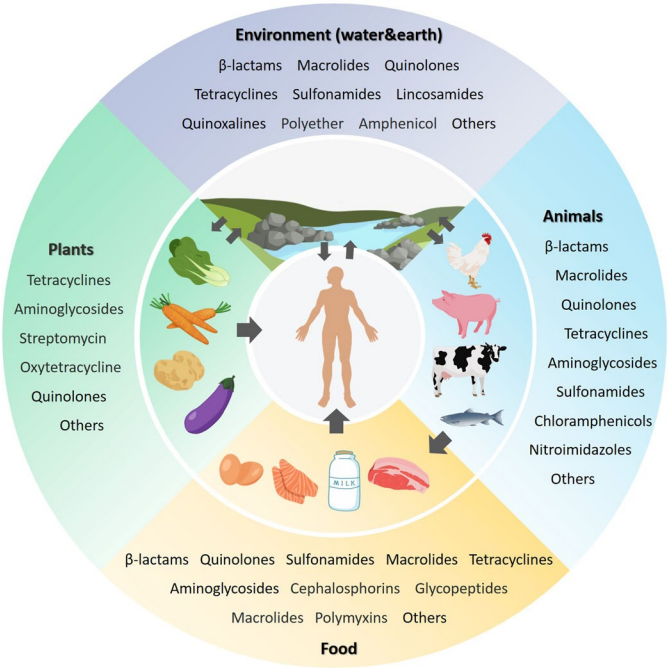

Figure 1. Scheme illustration of antibiotics circulating through environment, plants, animals, food and human body.

including enzyme-linked immunosorbent assay (ELISA) (Li et al. 2008), fluorescence immunoassay (Song et al. 2015), radioimmunoassay (Tao et al. 2012), colloidal gold immunoassay (Wang et al. 2017), and chemiluminescence immunoassay (Chen et al. 2016b). Although immunoassay is simple and specific, its application is limited due to the difficulty in antibody preparation, limited antibody types, and the easy cross-reaction.

Aptamer technology for antibiotics detection

So far, dozens of antibiotic aptamers have been successfully selected and applied into antibiotic detection, including penicillin G (Paniel et al. 2017), kanamycin (Song et al. 2011), tobramycin (González-Fernández et al. 2011), neomycin (Groher and Suess 2016), streptomycin (Tereshko, Skripkin, and Patel 2003), chloramphenicol (Duan et al. 2016), ampicillin (Song et al. 2012), enrofloxacin (Dolati et al. 2018), ciprofloxacin (Jaeger et al. 2019), ofloxacin (Ben Aissa et al. 2020), tetracycline (Chen et al. 2014), metronidazole (Wei et al. 2020), etc. Recently, Sun, Zhao, and Liang (2021) summarized the application of sensors and nanomaterial technology to detect antibiotic residues in food and the environment, introduced the aptamer biosensors in a large amount of detail and gave high expectations to future development prospects. Meanwhile, Yue et al. (2021) systematically summarized the application of optical and electrochemical aptamer biosensor for the detection of aminoglycoside antibiotics, such as tobramycin, neomycin, kanamycin, streptomycin, etc.

Although aptamer technology has drawn extensive attention, it still has some disadvantages. For example, long-chain aptamer may possess some redundant or unnecessary bases that cause steric hindrance which influence its affinity and specificity, or forming adverse secondary structure and interact with complex matrix, resulting in false positive or nonspecific signals (Debiais et al. 2020; Ye et al. 2019).

In order to avoid the disadvantages and improve the performance of aptamer-based biosensors, in recent years, various technologies were investigated to optimize the aptamer sequences, for example truncation, mutation, splitting, etc. (Jia et al. 2020; Sun et al. 2019; Wang et al. 2019). Among these technologies, the splitting of aptamer sequence is particularly worth noting given to its outstanding performance in small molecular target detection.

Overview of split aptamers

Concept of split aptamers

Split aptamers were obtained by splitting the optimized parent aptamer or truncated aptamer into two or three fragments, which have the same characteristic of identifying the original target when acting together. This split strategy was first reported by Stojanovic, de Prada, and Landry (2000). They innovatively split cocaine and rATP aptamers into two fragments, respectively. After the splitting, the length of the fragment was 16 bp and 27 bp for cocaine aptamer and 21 bp and 20 bp for rATP aptamer. Their studies showed that the two fragments formed after the splitting could still bind to their corresponding targets specifically, and a self-assembled fluorescence biosensor was successfully constructed for detection. This research opened up a new field of aptamer-based biosensor design and initiated the study of split aptamers.

Pros and cons of split aptamers

Compared with normal aptamers, split aptamers possess obvious advantages. Firstly, aptamers after splitting have a shorter length, with each strand generally no more than 30 bases, which is conducive to biosynthesis and cutting cost. In particular, when optimizing the concentration of aptamer for the detection, adjustment of only one split is needed instead of the whole parent aptamer sequence, thus reducing the cost (Kolpashchikov and Spelkov 2021). Secondly, split aptamers have a simple structure, which can help to avoid the generation of adverse secondary structure and is conducive to the specific binding with the target. Adopting split aptamers in biosensors can not only improve the sensitivity of detection but also avoid the generation of false positive or false negative results (Chen, Yan, and Yang 2016a). For example, a dual-color fluorescent colocalization method was established to detect adenosine triphosphate (ATP) using split aptamers. The detection limit was down to 100 fM and linear range of 1 pM to 5 nM, showing distinctly higher sensitivity as compared to previously reported methods

(Zhang et al. 2018). Thirdly, the biosensor design using split aptamers is more flexible. The design of split aptamer biosensor was originally inspired by the assembly of the heavy and light chains of an Fv antibody fragment (variable fragment) (Stojanovic, de Prada, and Landry 2000). Based on the ligand-dependent binding of aptamer fragments and target, the split aptamer can be used to develop a sandwich-type biosensor (Zhu et al. 2016), which enriches the form of aptamer biosensors.

Nevertheless, split aptamers also have some limitations, such as easily degraded by nucleases, difficulty in splitting the parent aptamer and the stability of split aptamer-target ternary complexes is hard to predict, which need further investigation.

Overall, on the basis of its advantages, the split aptamer-based biosensors are highly efficient to be used in the detection of small molecular targets like antibiotics.

Antibiotics targeted split aptamers

Following Stojanovic's successful acquisition of two split aptamers for cocaine and other drugs, early studies mainly focused on the construction of different biosensors for detection application using aforementioned split aptamers (Ma et al. 2019; Morris et al. 2018; Wu et al. 2013). Later, the research and development of small molecules targeted split aptamers attracted extensive attention. Over 260 papers related to split aptamer can be retrieved from the Web of Science database. Although only 17 small molecular targeted split aptamers have been successfully split so far (Qi et al. 2020), there are 6 split aptamers for antibiotics, accounting for up to 35%. As shown in Table 1, the reported antibiotic split aptamers are for enrofloxacin, kanamycin, tetracycline and tobramycin, neomycin and streptomycin detection, among which only enrofloxacin and kanamycin split aptamers are ssDNA, while others are RNA aptamers, the length of the split aptamers are around 11–33 bp, mostly around 15 bp.

The currently established split aptasensor are also summarized in Table 1, along with the detection performance and detection time. As can be seen, the currently reported split aptasensors are mostly nanoparticle-based sandwich-type colorimetric sensors (with exception). The LOD and detection range are mostly on the ng/L to μg/L scale and the detection time varies from 5 min to 6 h. Interestingly, although the Cu(I) catalyzed azide alkyne cycloaddition reaction (CuAAC) based fluorescence assay for kanamycin detection appears to achieve the lowest detection limit, its testing time is the longest (6 h). Whereas the other AuNPs aggregation induced-colorimetric aptasensor for kanamycin detection is much more rapid but less sensitive. The balance of detection time and sensitivity is indeed worth weighing. One of the aptasensors particularly worth mentioning is the TdT-gold nanoparticles (AuNPs) based lateral flow strip. This method not only have a relatively wide detection range, short detection time (5 min), but also extremely easy to operate and can be simply read by a smart phone application (APP).

Acquisition and mechanism of split aptamers

Despite the many advantages of split aptamers, the mechanism of splitting aptamers is still in the exploratory stage. Theoretically, split aptamers are acquired by segmenting a parent aptamer sequence. The binding ability of split aptamers was attributed to multiple factors including base paring, stacking effects, halogen bounding, hydrogen bonds, hydrophobic interactions and Van der Waals forces (Ben Aissa et al. 2020; Yue et al. 2021).

Split aptamer assembly principle

The basic principle of split aptamer assembly was as follows. In the absence of target, the enthalpic gain from base pairing and base stacking is lower than the entropic cost of assembly, therefore not generating aptamer-target complex. When added with the target ligand, the enthalpic gain increases due to the interaction between the ligand and the split aptamers and exceeded the entropic cost of assembly, making the thermodynamic balance moving toward assembly. Therefore, one of the key elements in designing the optimal split aptamers is to adjust the number of paired bases between the splits to the edge of assemblance (Neves et al. 2010a; Sharma and Heemstra 2011). Several technologies can be used to quantify the ligand-binding affinity and compare the thermodynamics of various designed sequences, for example, micro-scale thermophoresis (MST) (Volz et al. 2020), affinity capillary electrophoresis (ACE) (Liu et al. 2009), surface plasmon resonance (SPR) (Hasegawa et al. 2008), AuNP-based colorimetry (Liu et al. 2014), isothermal titration calorimetry (ITC) (Pagano, Mattia, and Giancola 2009), etc. The nuclear magnetic resonance spectroscopy (NMR) can also be utilized to analyze the stability and binding ability of the aptamers and provide structural information (Sakamoto 2017). By splitting, truncating and altering the parent aptamer sequences and testing the molecular interaction between target and the designed split aptamers, the optimal split aptamers can be obtained (Li et al. 2020).

Splitting mechanisms

The most challenging part of split aptamer design is to split parent aptamer at appropriate site. Limited work has been reported to articulate the splitting mechanisms.

In earlier studies, the bases that participate in binding to targets are considered essential and need to be reserved (Wang et al. 2019). Whereas the nonessential bases that do not interact with target molecule nor act as the contact supporting role may form unwanted tertiary structures and reduce the affinity to target (Jia et al. 2020). Therefore, the nonessential bases should be chosen as splitting sites (Jia et al. 2020). For example, the essential bases for streptomycin-binding RNA aptamer was reported to be at the two asymmetric internal loops (Wallace and Schroeder 1998; Windbichler and Schroeder 2006). Tereshko et al. split

Table 1. Currently reported antibiotic split aptamers and relevant biosensors.

				Split aptamer-based detection				
Antibiotics	Molecular formula/MW	Aptamer type	Sequences of split aptamers (5′ to 3′)	Aptasensor	LOD	Detection range	Detection time	Reference
Enrofloxacin	$C_{19}H_{22}FN_3O_3$/359.4	ssDNA	Split1 (32 bp): CCCATCAGGGGGCTAGGCT AACACGGTTCGGC Split2 (28 bp): TCTCTGAGCCCGGG TTATTTCAGGGGGA	Terminal deoxynucleotidyl Transferase (TdT)-Rolling Circle Amplification (RCA) based colorimetric split aptasensor	2.5 ng/L	1 ng/L–1 mg/L	2.5 h	Du et al. 2019
				TdT-gold nanoparticles (AuNPs) based lateral flow strip	0.1 μg/L	0.1 μg/L–0.1 g/L	5 min	Tian et al. 2020
Kanamycin	$C_{18}H_{36}N_4O_{11}$/484.5		Split1 (11 bp): TGGGGGTTGAG Split2 (10 bp): GCTAAGCCGA	Cu(I) catalyzed azide alkyne cycloaddition reaction (CuAAC) based fluorescence assay	2.2 ng/L	3.4 ng/L–1.7 μg/L	6 h	Belal et al. 2018
				AuNPs aggregation induced-colorimetric split aptasensor	1.7 μg/L	2.1–67.6 μg/L	55 min	Qi et al. 2022
Tetracycline	$C_{22}H_{24}N_2O_8$/444.5	RNA	Split1 (18 bp): GAGGGAGAGGUGAAGAAU Split2 (33 bp): ACGACCACCUAGGUAUA CCUAAAACAUACCCUC	N/A	N/A	N/A	N/A	Wang et al. 2019
Tobramycin	$C_{18}H_{37}N_5O_9$/467.5		Split1 (13 bp): GGGACUUGGUUUA Split2 (13 bp): GGUAAUGAUGCCC	Split aptamer-based enzyme-linked apta-sorbent assay (ELASA)	67.5 μg/L	N/A	3 h	Wang et al. 2019
Neomycin	$C_{23}H_{46}N_6O_{13}$/614.6		Split1 (12 bp): GGACUGGGCGAG Split2 (11 bp): AAGUUUAGUCC	N/A	N/A	N/A	N/A	Wang et al. 2019
Streptomycin	$C_{21}H_{39}N_7O_{12}$/581.6		Split1 (22 bp): GGAUCGCAUUUGGACUUCUGCC Split2 (18 bp): CGGCACCACGGUCGGAUC	Evanescent wave fluorescent (EWF) biosensor	2.7 μg/L	4.9–42.9 μg/L	5 min	Tereshko, Skripkin, and Patel 2003; Nick et al. 2016

this aptamer at the hairpin ring which was not involved in antibiotic recognition and obtained subsequent 18-mer and 22-mer RNA (Tereshko, Skripkin, and Patel 2003; Piganeau and Schroeder 2003).

For aptamers with unclear target binding pockets, secondary structure analysis and molecular docking (MD) can be adopted to help understand the aptamer-target complex structure (Navien et al. 2021). Hairpin-loop structure is one of the easiest and most common secondary structures for nucleic acid aptamers. According to Patel et al., the small molecule targets usually bind to the loop region (Patel 1997), but Stojanovic et al. proved that the loops are not always the binding site (Stojanovic, de Prada, and Landry 2000), the binding pocket can also be at the stem region (Huizenga and Szostak 1995). Parent aptamers with a three-way junction architecture are considered a "privileged architecture" since they usually comprise two "splittable" sites far away from the binding pocket. For example, the cocaine aptamer has a typical three-way junction architecture, the complex structure has been elucidated by NMR and the binding pocket was found to be at the internal loop region (Neves, Reinstein, and Johnson 2010b). Consequently, this aptamer was successfully split at the end bulge area and slightly modified to avoid interstrand binding (Wang et al. 2006). Moreover, the general splitting and engineering strategy of these three-way junction aptamers has been reported in Kent's work (Kent, Spiropulos, and Heemstra 2013).

Despite the traditional consensus, Wang et al. (2019) successfully split tetracycline, tobramycin, and neomycin-targeted aptamers at functionally essential sites. This study not only overthrown the traditional thinking, but also reflected our lack of knowledge in the mechanism of aptamer-target binding and pointed out new directions of split aptamer design.

Antibiotic aptamer splitting

Up to now, successfully acquired antibiotic aptamers include kanamycin, tobramycin, neomycin, streptomycin, tetracycline and enrofloxacin targeted aptamers. The secondary structure of kanamycin (Song et al. 2011), tobramycin (Jiang and Patel 1998) and neomycin (Wang et al. 2019; Ilgu et al. 2014; Jiang et al. 1999) aptamers are hairpin structure, streptomycin (Tereshko, Skripkin, and Patel 2003; Piganeau and Schroeder 2003; Nick et al. 2016) aptamer an asymmetric stem loop, tetracycline (Wang et al. 2019; Hetzke et al. 2019) aptamer a three-way junction architecture and enrofloxacin (Sha et al. 2021) aptamer a complex stem loop structure (Figure 3). The binding pocket were all reported to be at loop or bulge area of the parent aptamer. For the

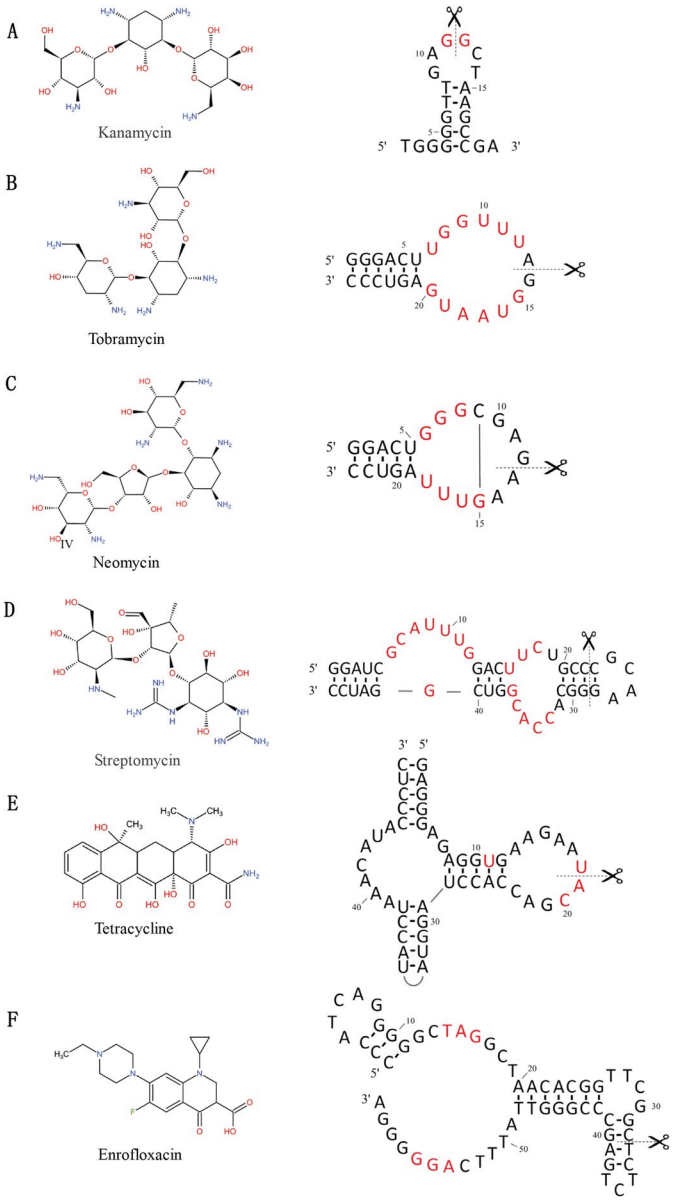

Figure 2. Chemical structure of antibiotics, corresponding aptamer splitting sites and binding essential nucleotides (marked in red). (A) Kanamycin; (B) Tobramycin; (C) Neomycin; (D) Streptomycin; (E) Tetracycline; (F) Enrofloxacin.

parent aptamers with hairpin or stem loop structure, the splitting sites were found to be at the symmetric end loop. The splitting site of tetracycline aptamer was at the binding essential end ring. And enrofloxacin aptamer splitting site was at a stem away from binding pocket.

Molecular dynamics simulations were conducted to investigate the hydrogen bond interactions between the streptomycin molecule and its' split aptamer (Figure 3B) (Nick et al. 2016). The 2′-OH of the uracil 11 (U11, blue) on the split 1 was found to form hydrogen bond with the NH group on the streptomycin. Guanine 12 (G12, gray) formed two hydrogen bonds with the OH group and NH group. Uracil 16 (violet) paired with the OH group, U17 (rose) paired with the NH_2 group. The N^7 of G110 (orange) and G111 (yellow) on split 2 formed hydrogen bond with NH_2 group respectively. In conclusion, the bases participating in the hydrogen bond formation were mainly guanine and uracil. This pattern holds in other antibiotic split RNA aptamers as well (Figure 2), especially for aptamers targeting antibiotics with NH/NH_2 groups.

Among the six reported antibiotic parent aptamers, 4 of them were split at binding essential pocket (kanamycin, tobramycin, neomycin and tetracycline aptamer), and 3 of which were aminoglycosides targeted aptamers (Figure 2). Jiang and Patel (1998) analyzed the tobramycin-aptamer complex and proposed potential intermolecular interaction (Figure 3D). The tobramycin molecule possesses 3 rings including one non-sugar ring and two amino sugar rings (Figure 3A). The two NH^{3+} group on non-sugar ring I was predicted to pair with the N^7 atom of G9, exocyclic oxygen of phosphate (O1P) of G8 and O1P oxygen of U10, the 2′-NH^{3+} group on the amino sugar ring II was predicted to pair with the O^4 atom on U11 and U12 and the O1P oxygen of G15, and the 3′-NH^{3+} group on the amino sugar ring III was predicted to pair with the O^4 of U7 and U19 on the RNA (Jiang and Patel 1998). The donor atoms like nitrogen of NH^{3+} group on tobramycin interact with acceptor atoms like nitrogen of G and oxygen of U on the aptamer, potentially forming intermolecular hydrogen bonds. These hydrogen bonding interactions involve each of the NH^{3+} groups on tobramycin to anchor the target tobramycin molecule within the RNA binding pocket (Jiang and Patel 1998). Another work of Jiang et al. analyzed the neomycin-aptamer complex (Figure 3C) (Jiang et al. 1999). Similarly, the 1′-NH^{3+} and 3′-NH^{3+} groups on non-sugar ring I were predicted to form potential hydrogen bonds with the O^4 acceptor atoms of U21, O^6 atom of G9 and the N^7 of G10, the 2′-NH^{3+} group on amino sugar ring II predicted to pair with the O^4 of U19 and O6 and N7 of G18, and the 6′-NH^{3+} group on ring II paired with the O1P oxygen of G9. These evidences suggest the amino groups have strong effect on molecule-RNA binding, especially with guanine and uracil. This strong intermolecular interaction may also explain the successful splitting of the aptamer at binding pocket.

Overall, amino groups on antibiotics have an important role in binding to their aptamers. Antibiotics with amino groups tend to form hydrogen bonds with their RNA aptamers at guanine and uracil bases. Aptamers of amino group-containing antibiotics seem more likely to be successfully split, and the splitting sites are less restricted by the "no splitting at binding pocket" rule, probably due to the strong bonding force between the amino group and the guanine and uracil bases.

Application of split aptamer in antibiotic detection

Enrofloxacin (ENR)

The Split1 and Split2 chains of enrofloxacin (ENR) split aptamers are 32 bp and 28 bp, respectively. Du et al.(Du et al. 2019) constructed a sandwich-type aptamer biosensor based on the terminal deoxynucleotidyl transferase (TdT) and rolling circle amplification (RCA) to realize the sensitive detection of enrofloxacin (Figure 4A). The Split1 and Split2 were immobilized on gold nanoparticles and magnetic beads through sulfhydrylation and amination, respectively. A ploy

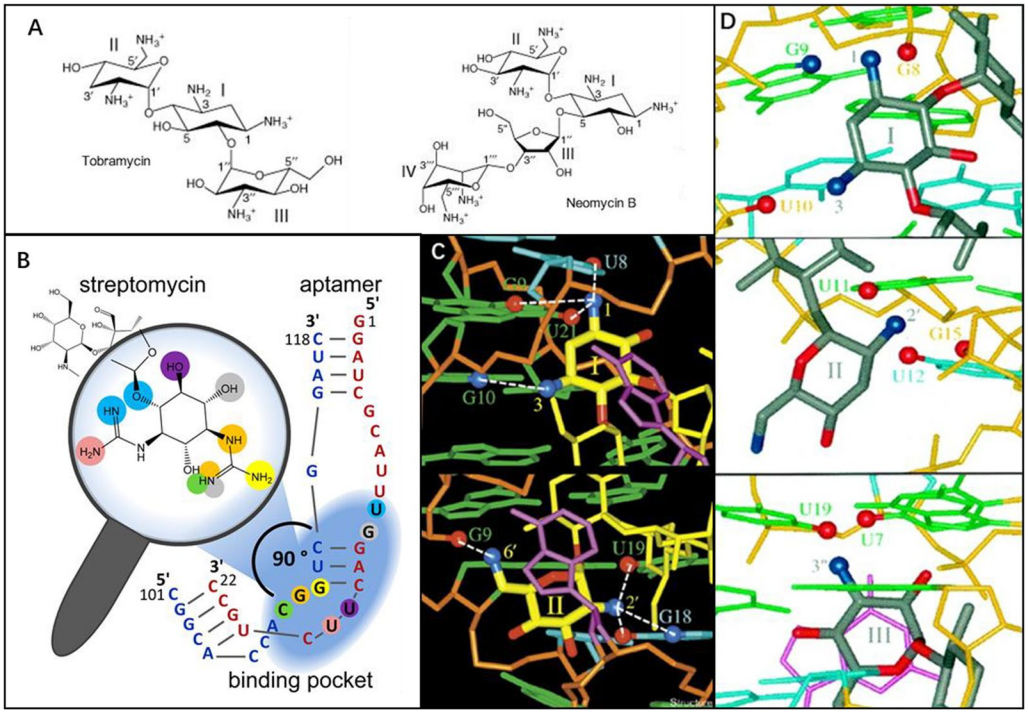

Figure 3. (A) Chemical formula and numbering system of tobramycin and neomycin. (B) Scheme of the streptomycin split aptamer (Nick et al. 2016). (C) Intermolecular alignments and hydrogen bonding of neomycin and its aptamer (Jiang et al. 1999). (D) Intermolecular alignments and hydrogen bonding of tobramycin and its aptamer (Jiang and Patel 1998).

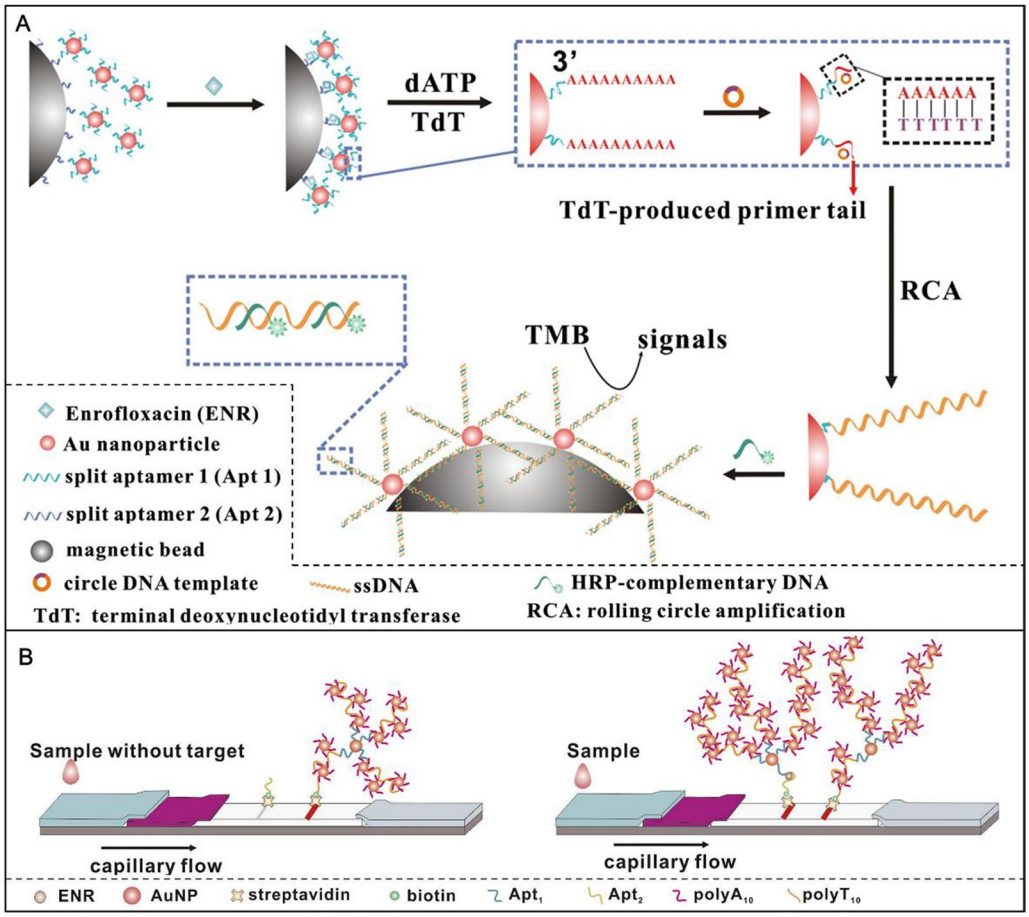

Figure 4. (A) Schematic illustration of TdT-RCA assay for enrofloxacin detection (Du et al. 2019). (B) Schematic of the split aptamer-based lateral flow strip for enrofloxacin detection (Tian et al. 2020).

adenine deoxynucleotide (A) tail was added to the 3′ end of Split1 sequence for immobilization as a primer for the rolling circle amplification. The magnetic beads were used for rapid separation and enrichment of the targets. In the presence of enrofloxacin, two split aptamers specifically bound to ENR to form a sandwich structure. After magnetic separation and enrichment, TdT catalyzed the generation of poly A sequences, which were used as a primer to initiate the RCA. The amplified ssDNA was then hybridized with complementary horse radish peroxidase (HRP) DNA strand, and finally substrate tetramethylbenzidine (TMB) was added to activate the oxidation reaction and generate colorimetric signal, thereby realizing quantitative detection of ENR. Combining the technology of split aptamer, magnetic separation and RCA resulted in lower limit of detection (LOD) of ENR (2.5 pg/mL) with a linear range of 1 pg/mL–1 μg/mL. The recovery rate of spiked fish samples was between 87.5% and 92.1%, which showed good feasibility. In addition, based on this principle, the authors also realized the simultaneous detection of four substances including ENR, Pb^{2+}, *Escherichia coli* O157:H7 and tropomyosin, which provides a reference for the simultaneous detection of multiple targets by split aptamer biosensor.

Tian et al. (2020) also used ENR split aptamers combining with gold nanomaterials and TdT to develop a test strip that can be read by smartphone application (APP) (Figure 4B). Since RCA technology was not introduced, the detection limit of this test strip was only 1 mg/mL. However, this product is more rapid for detection and easier to be promoted in combination with smart phones, therefore it has a good market application prospect.

Kanamycin (KANA)

Kanamycin aptamers are short-chain ssDNA aptamers that include a 11 bp split and a 10 bp split. Inspired by click chemistry, Belal (Belal et al. 2018) et al. adopted kanamycin split aptamers and developed a fluorometric detection method based on the Cu(I) catalyzed azide-alkyne cycloaddition reaction (CuAAC) to achieve accurate and sensitive detection of kanamycin. Split 1 was labeled with $-NH_2$ at the 3′ end to couple with -COOH tagged CuS nanoparticles and the Split 2 was labeled with biotin at the 5′ end to be immobilized on streptavidin-coated magnetic beads through streptavidin-biotin interaction (Figure 5A). The addition of kanamycin generates the formation of

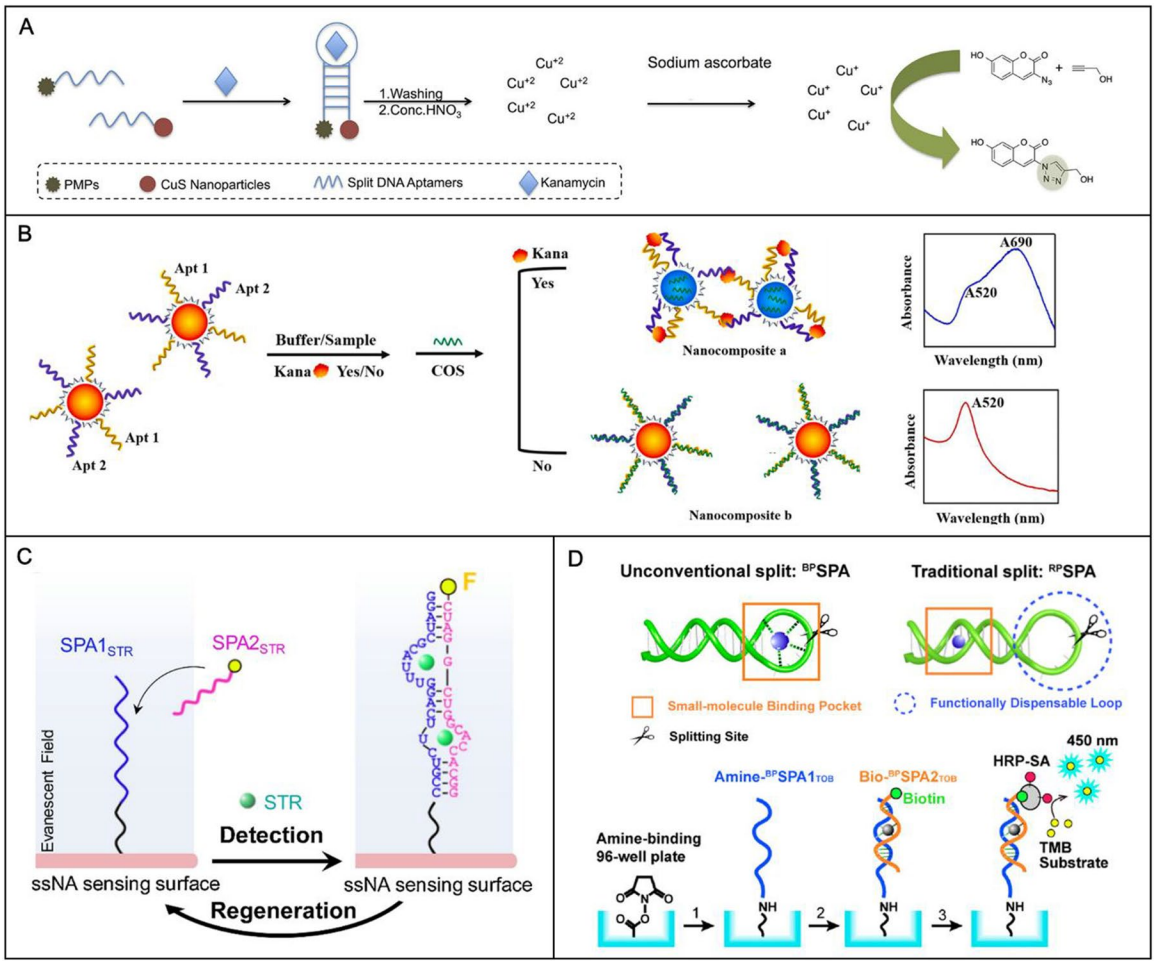

Figure 5. (A) Schematic of CuAAC click reaction based split aptasensor for kanamycin detection (Belal et al. 2018). (B) Schematic of AuNPs aggregation induced-colorimetric split aptasensor detecting kanamycin (Qi et al. 2022). (C) Schematic of split aptamer-based sandwich-type biosensor for detection of streptomycin (STR) (Zhu et al. 2021). (D) Schematic illustration of engineering tetracycline split aptamers from the parent aptamer at unconventional and traditional splitting site and the split aptamer based ELASA for tobramycin detection (Wang et al. 2019).

sandwich complexes, and the complexes were separated via magnet. Sodium ascorbate was added to reduce Cu(II) to Cu(I) and activate the click reaction between fluorogenic 3-azido-7-hydroxycoumarin and propargyl alcohol to generate a fluorescence signal by 1,4-disubstituted-1,2,3-triazole. Their study showed good linearity between the fluorescence intensity and the logarithm of kanamycin concentration in the range of 0.04–20 nM, and the detection limit was down to 26 pM. Their study also showed good selectivity and the recovery rate of human serum samples was over 95.6% with relative standard deviations (RSD) of 1.73%–2.09%. However, this method requires at least 6 h to get the result, which is not the best performance for an aptasensor.

Recently, Qi et al. have developed a new aptasensor based on the same split aptamer to detect kanamycin (Qi et al. 2022). In this research, an AuNPs aggregation induced-colorimetric aptasensor was established. The two split aptamers (Apt 1 and Apt 2) were added with poly A tails and adsorbed onto the AuNPs through ploy A-Au interaction. As shown in Figure 5B, when kanamycin presence, the Apt 1 and Apt 2 specifically recognize the target and form ternary complex, resulting in the aggregation of AuNPs and form nanocomposite a. Chitosan oligosaccharide (COS) was used as the AuNPs aggregation inducer as it can cause the electrostatic stability of AuNPs to decrease, and change the color of the solution from wine red to purple. Whereas when the target absence, the positively-charged COS would bound to the negatively-charged aptamers instead of the AuNPs surface, generating nanocomposite b, which would not induce the color change. This strategy achieved a LOD of 20.58 nM, and a linear range of 25–800 nM. Furthermore, Qi et al. verify the accuracy and repeatability of this aptasensor in real samples and got good recoveries (98.49%–104.9% and 85.69%–107.0%). Although less sensitive, this aptasensor detection require only 55 min, is much more rapid as compared to the Belal's sensor. Qi et al. have achieved great improvement since Belal et al., for future development, more rapid, concise and straightforward detection method like lateral flow strip is expected.

Streptomycin (STR)

Split aptamers against streptomycin are RNA in nature, including a 22-mer and a 18-mer RNA strand (Nick et al. 2016). Based on the previously selected split aptamers (Tereshko, Skripkin, and Patel 2003; Nick et al. 2016) with high sensitivity and selectivity, Zhu et al. (2021) established an evanescent wave fluorescent (EWF) biosensing platform. The mechanism of this biosensor is shown in Figure 5C. One of the splits ($SPA1_{STR}$) was labeled with -NH_2 and 6 carbons at the 5′ end and immobilized on the surface of a modified optical fiber with -CHO groups. The -NH_2 groups allowed $SPA1_{STR}$ to covalently bind to the fiber and the 6 carbons allowed the $SPA1_{STR}$ to expose its structure completely. The other split ($SPA2_{STR}$) was labeled with Cy5.5, which can only be excited when bound to the optical sensing surface. Therefore, when incubating the $SPA1_{STR}$ and $SPA2_{STR}$ with streptomycin, the sandwich-type complexes formed, the Cy5.5 on $SPA2_{STR}$ was excited by the evanescent field and emit fluorescence signals, which could be record and quantified by a built-in computer. This detection platform was well proven and its sensitivity, specificity and reliability have been verified with LOD of 33 nM and linearity range between 60 nM and 526 nM. Recovery study was also conducted using real-water samples and acquired a satisfactory accuracy with recovery rates of streptomycin ranged from 79.2% to 96.8%. Moreover, the $SPA2_{STR}$ and STR can be washed off from the sensing surface simple with SDS buffer, and the optical sensing fiber can be regenerated to detect new samples. The reported study has reused the fiber 100 times and maintained good regeneration performance. This novel detection platform is sensitive, specific, regenerable, rapid and easy to operate, exhibiting high potential in application in automatic and on-line monitoring for targets.

Tetracycline (TC), tobramycin (TOB) and neomycin (NEO)

It was generally recognized that the integrity of conserved motifs and the proximity of essential nucleotides need to be reserved during the splitting of parent aptamers. According to this rule, tetracycline aptamer along with aptamers against tobramycin, neomycin, histidine and many others were regarded as "unsplittable" due to the lack of functionally dispensable site. However, Wang et al. (2019) have overthrown this conventional theory by splitting at the functionally essential sites and successfully obtained the split aptamers against tetracycline, tobramycin and neomycin. Their study designed an unconventional split aptamer with Broken initial small-molecule binding Pockets (^{BP}SPA, Figure 5D) based on an engineered three-way junction aptamer against tetracycline. And explored the performance of the unconventional split aptamer by comparing to the conventional split aptamer with Reserved initial small-molecule binding Pockets (^{RP}SPA, Figure 5D) and wild-type aptamer. The dissociation constant ((K_d)) of $^{BP}SPA_{TC}$ was 101.5 ± 0.7 nM, showing a slightly higher affinity against tetracycline comparing to wild-type aptamer ((K_d) = 130.5 ± 10.6 nM), which was speculated to resulted from the pre-organized structure and conformational flexibility of the split aptamer. The $^{BP}SPA_{TC}$ complexes were analyzed by fluorescence resonance energy transfer (FRET) assay and MD simulations, results suggest the ^{BP}SPA formed a new binding pocket in the presence of its target, which justified their hypothesis of the feasibility of splitting at functionally essential sites. Following this strategy, they also successfully engineered the split aptamers for tobramycin and neomycin.

Based on the newly split aptamer, they developed an enzyme-linked apta-sorbent assay (ELASA) to detect tobramycin (Figure 5D). For the novel ELASA assay, amine-$^{BP}SPA1_{TOB}$ was covalently conjugated to an amine-binding 96-well plate. The $^{BP}SPA2_{TOB}$ was biotinylated and can capture the peroxidase-streptavidin (HRP-SA) conjugate through biotin-streptavidin interaction, which can

then catalyze the chromogenic substrate to form yellow color. When TOB presence, the TOB, $^{BP}SPA2_{TOB}$ and $^{BP}SPA1_{TOB}$ form ternary complex and immobilized onto the plate, while otherwise the $^{BP}SPA2_{TOB}$ can be easily washed off without the TOB, resulting in no color development. A LOD of 1 μM toward TOB was achieved by this ELASA, and this performance is quite impressive comparing to the old parent aptamer-based electrochemical sensor, as it shows much higher signal change in the presence of only 1 μM of TOB (Schoukroun-Barnes et al. 2014).

In sum, this study gave us a better understanding of the mechanism of the split aptamer design and the established split aptamer-based ELASA is well-performed, simple in both design and operation and can be extended to other split aptamer-based detection. However, the fly in the ointment is that they did not apply this assay to the detection of other two newly split aptamers (toward tetracycline and neomycin), and we were not able to find the application of these two split aptamers in the current state.

Conclusion

Food safety problems caused by antibiotics are a serious threat to human health and has become the focus of attention for people from all walks of life. Screen excellent molecular probes and develop sensitive, rapid, economical and efficient method for antibiotic detection is an inevitable trend to replace the traditional assays. Novel split aptamer-based biosensor has attracted increasing attention in this field. Because split aptamers have smaller molecules compared to their parent aptamers and possess conformational flexibility, which makes it them less prone to form unwanted structures and generate false positive or nonspecific signals.

Although only 6 reported antibiotics aptamer were successfully split so far, including 4 aminoglycoside antibiotic targeted aptamers, they accounted for a large proportion of the total splitting aptamers. With so many different kinds of antibiotics, the most important challenge to the future is to reveal the split mechanism and discover more antibiotic split-aptamers. The second urgent challenge to the future is to improve the stability of the splits-target complexes. The third is devote to work out the recognition mechanism of split aptamer with its target. Finally, split aptamer-based quantification approaches like fluorescence, colorimetric, electrochemical, chemiluminescent and other novel methods along with all kinds of signal amplification measures are needed for further investigation to develop practical and reliable detection methods for antibiotic targets.

Declaration of interest statement

The authors declare that there is no conflict of interest.

Funding

This work was financially supported by the Natural Science Foundation of Jiangsu Province (BK20201452), Nanjing Pukou District Social Development Project (S2020-17), the National Natural Science Foundation of China (31871881), Jiangsu Agricultural Agriculture Science and Technology Innovation Fund (CX (18) 2025), the Distinguished Professor Program of Jiangsu Province.

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