Review Article

Recent Advances in Aptamer- Based Biosensors for Detection of Antibiotic Residues

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Abstract | Antibiotics are widely used as bacteriostatic or bactericidal agents either to kill or inhibit the growth of microorganisms. Their abuse results in various side effects on the human health, environment and agriculture. The occurrence of bacterial "suprainfection" with tolerance to antibiotics has attracted the significant attention, mainly due to the consequences of multi drug resistance and potential threat to human health and the environment. With the increasing incidences of antimicrobial contamination, especially in food, dairy products, agriculture and environment, their regular monitoring is on prime interest. Aptamers are synthetic short sequences of single stranded (ss) oligonucleotides (ss-RNA or DNA), which are developed by an *in-vitro* selection process known as "Systematic Evolution of Ligands by Exponential Enrichment (SELEX)" technique. Among the receptors available for biosensing, aptamer exhibit the advantages of high specificity, selectivity, stability, facile labelling and modification, which makes them ideal candidates for development of new biosensing applications for detection of specific target molecules. In the present review, we concentrate on the recent advances in the development of aptasensors for antibiotics residue analysis based on electrochemical signal generation. Aptamers possesses the strong potential as receptors for the development of biosensors for antibiotics detection; therefore, a specially designed aptamer specific to an antibiotic may be suitable for this purpose. In this review, the importance of detection of antibiotic residue contamination, reported analytical methods, advancement in biosensing platform especially regarding electrochemical transduction are discussed in detail. Finally, future prospects toward the development of selective and sensitive aptasensors for antibiotic detection are presented.

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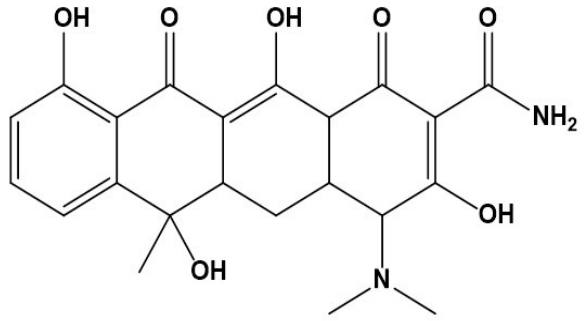
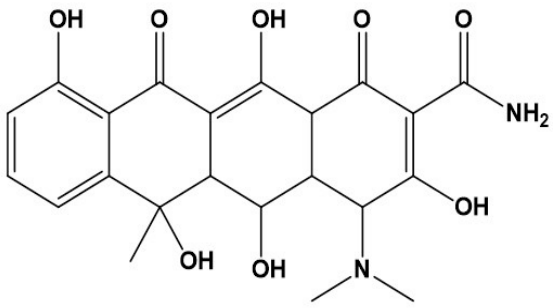
Due to the increasing prevalence and incidence of antibiotic residue contamination and drug resistance around the world, early and specific detection of antibiotic residues has garnered significant attention. The emergence of microorganisms resistant towards important antibiotics results in consistently high mortality rates (White et al., 2011; CDC, 2006). However, indiscriminate antibiotic use or abuse results in serious clinical infections, requiring aggressive therapy and as growth promoters in aquaculture and agriculture, antibiotics have become a global concern, due to the serious side effects and multidrug resistance affecting the human health and the environment (Rogatsky and Stein, 2005; Cabello, 2006; Dapra et al., 2013; Leung et al., 2013). Despite these problems, the increased incidences of "suprainfection" caused by

the secondary effects of antibiotics, ensuring the use of suitable amounts of antibiotics is probably the major health problem (Megoules and Koupparis, 2005). The presence of antibiotic residues such as aminoglycosides, tetracyclines, fluoroquinolones, chloramphenicol *etc*. (Figure 1) in food and food products causes the sides effects such as heapatotoxicity, ototoxicity, blue baby syndrome, teratogenicity, nephrotoxicity, decrease in growth and metabolism as well as increase in the mortality and morbidity rates (Oertal at al., 2004; Jin et al., 2006; Pilehvar et al., 2012; Pinacho et al., 2014; Le et al., 2016).

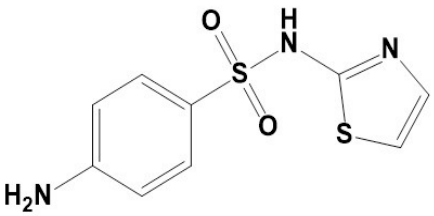
Normally, microorganism have very short generation times, thus they can quickly evolve resistance toward the applied antibiotics, if the antibiotic is present in sub-lethal concentrations (Gullberg et al., 2011). To ensure food safety, human health and minimise the development of resistance towards antibiotics, it is critically important to mandate limits on antibiotics use, and release into the environment. Various stringent limits for the presence of antibiotic residues in different food matrices have been fixed by regulatory agencies such as the United States Food and Drug Administration (USFDA), Food Safety and Standard Authorities of India (FSSAI), Codex Alimentarious Commission (CAC) and European Union (EU) as shown in the Table 1 (CAC, 2003; Suarez et al., 2009; Homem and Santos, 2011; Food Safety and Standard Authority of India, 2012).



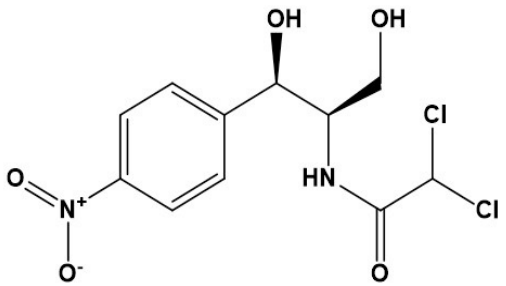
Tetracycline (TET)



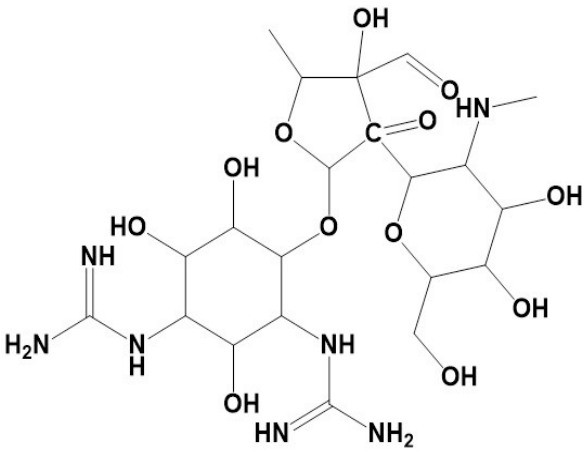
Oxytetracycline (OTC)



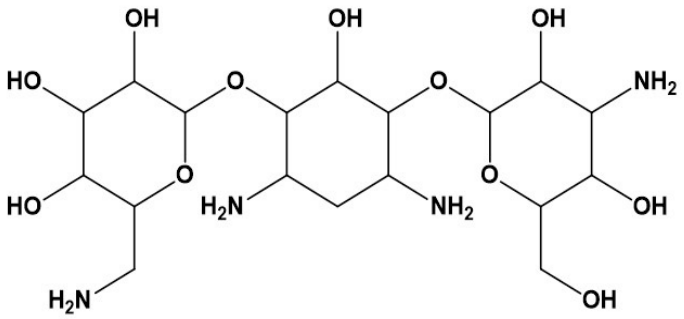
Sulphathiazole (SFZ)



Chloramphenicol (CAP)



Streptomycin (STR)



Kanamycin (KANA)

Figure 1: *Structures of common antibiotic residues found in food and environmental samples*

Table 1: *Regulatory maximum residue limit for various group of antibiotics residues*

Group of Antibiotics	Matrix	USFDA and FSSAI*	CAC**	EU***
Tetracyclines	Milk, milk products, meat, fruit juice	100-300 μg kg^{-1}	200 μg kg^{-1}	100 μg kg^{-1}
Aminoglycosides	Milk, milk products, meat, fruit juice and beverages	125 μg kg^{-1}	200 μg kg^{-1}	200 μg kg^{-1}
Fluoroquinolones	Milk, milk products	10–1900 μg kg^{-1}	10–400 μg kg^{-1}	100 μg kg^{-1}
β-lactum	Milk, milk products, meat	5 μg kg^{-1}	4 μg kg^{-1}	4 μg kg^{-1}

*: *Suarez et al. (2009), Food Safety and Standard Authority of India (FSSAI) (2012);* **: *Codex Alimentarious Commission (CAC) (2003);* ***: *Homem and Santos (2011);* **USFDA:** *Unites States Food and Drug Administration;* **EU:** *European Union*

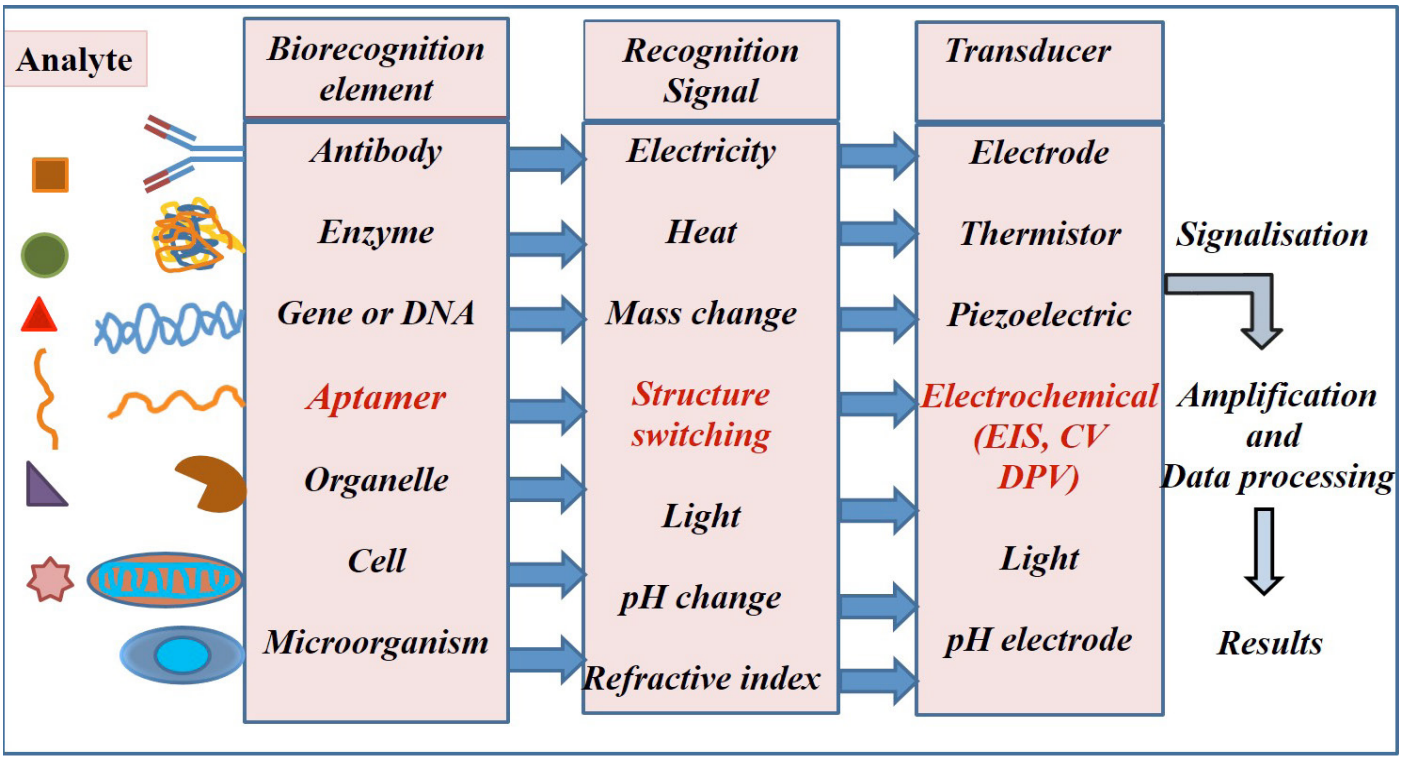


Figure 2: *Block diagram for the principle of a biosensor*

Therefore, it is critical to develop sensitive and selective analytical methods for regular monitoring and routine analysis of antibiotic residues in foods, clinical samples and the environment. Various conventional analytical techniques including high performance liquid chromatography coupled with ultraviolet detection (HPLC-UV) (Benito-Peña et al., 2006; Zhou et al., 2009; McWhinney et al., 2010), HPLC coupled with mass spectrometry (Aguilera-Luiz et al., 2008; Gaugain-Juhel et al., 2009), capillary electrophoresis (Vera-Candioti et al., 2010), liquid chromatography with mass spectroscopy (LC-MS/MS) (Cháfer-Pericás et al., 2011), surface-enhanced Raman spectroscopy (SERS) (Zheng and He, 2014), mid-infrared spectroscopy (Botelho et al., 2015), enzyme linked immunosorbent assay (Li et al., 2008; Le et al., 2009; Huang et al., 2010; Chen et al., 2013), immunosensor and surface plasmon based immunosensor (Adrian et al., 2008; Huang et al., 2010; Rebe-Raz et al., 2009) have already been reported for detection of antibiotics in various matrices. However, the disadvantages of the above mentioned methods such as time-consuming processes, expensive and tedious control, high consumption of reagents, susceptibility to interference and *in vivo* production of antibody production limits their utility and makes such methods difficult to adopt for high throughput and on-site analysis of target molecules (Sharma et al., 2016). Recently, some innovation and improvements in the development of immunoassays including the integration of nanoparticles and miniaturization of assay platforms have been reported (García-Fernández et al., 2014; Liu et al., 2016). Therefore, the development of simple, robust, selective, specific and fast detection methods based on the alternative aptamer recognition elements is of prime interest.

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Biosensors and Novel Biorecognition Element

In the recent years, biosensors have emerged as an alternative to conventional analytical methods. A biosensor is a device that capitalizes on a biological recognition element, which recognizes a selective target molecule on the basis of different signal outputs such as thermal, optical, piezoelectric, electrochemical etc. Compared to conventional analytical methods, biosensors are portable, easy to handle, quick, practical and the user does not require special skills (PAC, 1992). Molecular recognition elements are key for biosensors, since their binding affinity and specificity determine the performance of the biosensor (Figure 2). Initially, antibodies and enzymes were explored as recognition elements for biosensing and analytical applications, due to their selectivity towards a broad range of target molecules. However, the need for animal immunization (antibody production), thermal instability, shorter shelf lives, loss of activity on labelling and irreversible denaturation limited the wide utility of antibodies or enzyme-based bioassays (Radi, 2011). Therefore, it became important to seek out alternative recognition elements for development of robust platforms for biosensing applications. In the recent years, aptamers as alternatives to antibodies have attracted great attention due to their high selectivity and sensitivity towards their cognate target molecules. Aptamers are the short sequences of single-stranded (ss) DNA or RNA oligonucleotides obtained from the *in vitro* process known as Systemic Evolution of Ligands by Exponential Enrichment (SELEX) which selects and amplifies aptamers from very large and diverse random DNA or RNA libraries (Song et al., 2008).

Aptamers possess the capabilities of high affinity and specificity towards their target molecules. As recognition elements, aptamers also offer the advantages of greater selectivity, thermal stability, facile labelling and cost effective modification, which makes them ideal candidates for development of new biosensing methods for detection of specific target molecules (Swensen et al., 2009). Based on the above properties and the possibility of rationally designed sequences, the aptamers serve as effective recognition elements in the design and development of stable, inexpensive, robust and practical biosensing platforms for detection of target analytes. Despite the great promise of aptamer-based sensing strategies in the areas of clinical diagnostics and therapeutics only few aptamer-based products exist in the market (Bruno, 2015). The reason was attributed to the commitment of huge financial investment in humanized monoclonal antibodies and ignorance about aptamers and their performance in the research and development. To date, fewer companies have marketed aptamers as a component of aptamer assay kits such as the NeoVentures Ochratoxin A and aflatoxin ELISA-like microwell plate assays and affinity columns (Penner, 2012).

This review is focused on published aptasensors developed for detection of antibiotic residue contamination that has been designed based on electrochemical detection methods. In the presence of its target molecule, the aptamer folds into a specific 3D conformation. One well-known example of such a 3D confirmation is the antiparallel-G-quadruplex aptamer-target complex structure. This target-aptamer complex formation results in various signal generation depending upon the transduction principle applied. Electrochemical detection methods offer easy to modify, miniaturized, automated, disposable assays or sensors, high detection speed, cost effective and low volume consumption of reagents. Thus, based on these advantages, electrochemical aptasensors appear to be well-suited for practical and on-site applications. Hereafter, in this review, we are therefore focused on the development of electrochemical aptasensors for detection of antibiotic residues.

Recent Advancement in Electrochemical Aptasensors for Detection of Antibiotics

In previous years, electrochemical aptasensor devices have received considerable attention in connection with the transduction of aptamer-target interactions. In the design of a typical electrochemical based aptasensor, the immobilization of aptamer on the electrode surface is usually performed by thiol-gold affinity, diazonium coupling and click chemistry, where the analyte-aptamer event is monitored based on electrochemical signal generation (Adams et al., 2012; Hayat et al., 2013; Goud et al., 2016). The generation of electrochemical signals corresponds to the amount of analyte present. Electrochemical aptasensors offer the advantages of high sensitivity, selectivity, stability, compatibility with novel microfabrication, disposability, portability and miniaturization. Thus, these aptasensor are extremely attractive for transducing the aptamer recognition event in a simple, fast and inexpensive manner. Electrochemical aptasensors can be further divided into several groups based on the

assay format and method of detection. The electrochemical measurement can be performed in the form of differential pulse voltammetry, cyclic voltammetry, chronoamperometry, electrochemical impedance spectroscopy, or linear sweep voltammetry (Valimaa et al., 2010). A number of electrochemical aptasensor have been reported for antibiotics detection in various food matrices, which are discussed in Table 2. We will discuss step by step each strategy. Recently, Zhou et al. (2012) has been reported the development of electrochemical aptasensor for detection of tetracycline in spiked milk samples.

For construction of this aptasensor, a glassy carbon electrode surface was modified with functionalized multiwall carbon nanotubes (MWCNTs). Immobilization of anti-TET aptamer on the activated surface of modified GCE was carried out using EDC/NHS crosslinking chemistry. Under optimized experimental conditions, the differential pulse voltammetric (DPV) measurements were performed to quantify the amount of TET (Figure 3). The developed aptasensor showed a good sensitivity in the range 0.1 nM to 0.5 μM with a LOD of 5.0 nM. For practical feasibility of this aptasensor, the aptasensor performance was successfully demonstrated in spiked milk samples.

For construction of electrochemical aptasensors, signal amplification is a key factor. The integration of nanomaterial based strategies has promising potential in ultrasensitive detection due to their phenomenal properties. Due to their catalytic and high surface-area ratios, nanoparticles have gained attention in the design and development of new biosensing application (Sharma et al., 2016). Guo et al. (2015) has reported a novel electrochemical aptasensor for ultrasensitive detection of kanamycin. The aptasensor was fabricated based on MWCNTs–1-hexyl-3-methylimidazolium hexafluorophosphate ($HMIMPF_6$) (MWCNTs–$HMIMPF_6$) and NP-PtTi alloy as the matrix, which allow the effective immobilization of biorecognition matrix with an unblocked conductive pathway for electron transfer. The reported aptasensor exhibited a linear range of 0.05-100 ng mL^{-1}

Table 2: *Reported electrochemical based aptasensor for antibiotic detection*

Analyte	Detection Technique	Detection Range	Matrix	Limit of Detection	Ref.
Tetracycline	Differential Pulse voltammetry (DPV, glassy carbon electrode (GCE)	0.1 nM to 50 μM	Milk	5 nM	Zhou et al. (2012)
Kanamycin	DPV (GCE with MWCNT and alloy)	0.05–100 ng mL^{-1}	Milk	3.7 pg mL^{-1}	Gua et al. (2015)
Kanamycin	DPV	5 fM to 100 pM	Milk	1.3 fM	Wang et al. (2016)
Tetracycline	Electrochemical impedance spectroscopy (EIS)	5.0 to 5.0× 10^3 ng mL^{-1}	Milk	1.0 ng mL^{-1}	Chen et al. (2014)
		1.0×10^{-12} to 1.0×10^{-7}M	Milk	3.0×10^{-13}M	Jahanbani and Benvidi (2016)
		10 to 3000 ng ml^{-1}	Milk	10 ng ml^{-1}	Li et al. (2016)
Tobramycin	EIS (A displacement assay)	3.0 to72.1μM	Human Serum	1.8 μM	Gonzalez-Fernandez et al. (2011)
Ampicillin Kanamycin A	EIS (Interdigitated microelectrodes)	100 pM to 1 μM	Milk	10 pM	Dapra et al. (2013)
		10 nM to 1 mM	Milk	-	
Streptomycin	Cyclic voltammetry (CV)	30–1500 nM	Milk	14.1 nM	Danesh et al. (2016)
			Serum	15.3 nM	
Tetracycline	DPV	1×10^{-10} to 1×10^{-3} M	Milk	0.56×10^{-11} M	Guo et al. (2015)
Ampicillin	DPV	5 pM to 10 nM	Milk	1.09 pM	Wang et al. (2015)
		0.02 to 40 nM	Milk	4 pM	Wang et al. (2016)
Oxytetracycline (OTC)	Square wave voltammetry (SWV)	10 to 600 ng ml^{-1}	Blood Serum Urine	9.8 ng ml^{-1}	Zheng et al. (2013)
OTC	SWV	0.0005-50 ng mL^{-1}	Milk	0.10 ng mL^{-1}	Yan et al. (2016)
Chloramphenicol (CAP)		0.0005-50 ng mL^{-1}	Milk	0.15 ng mL^{-1}	

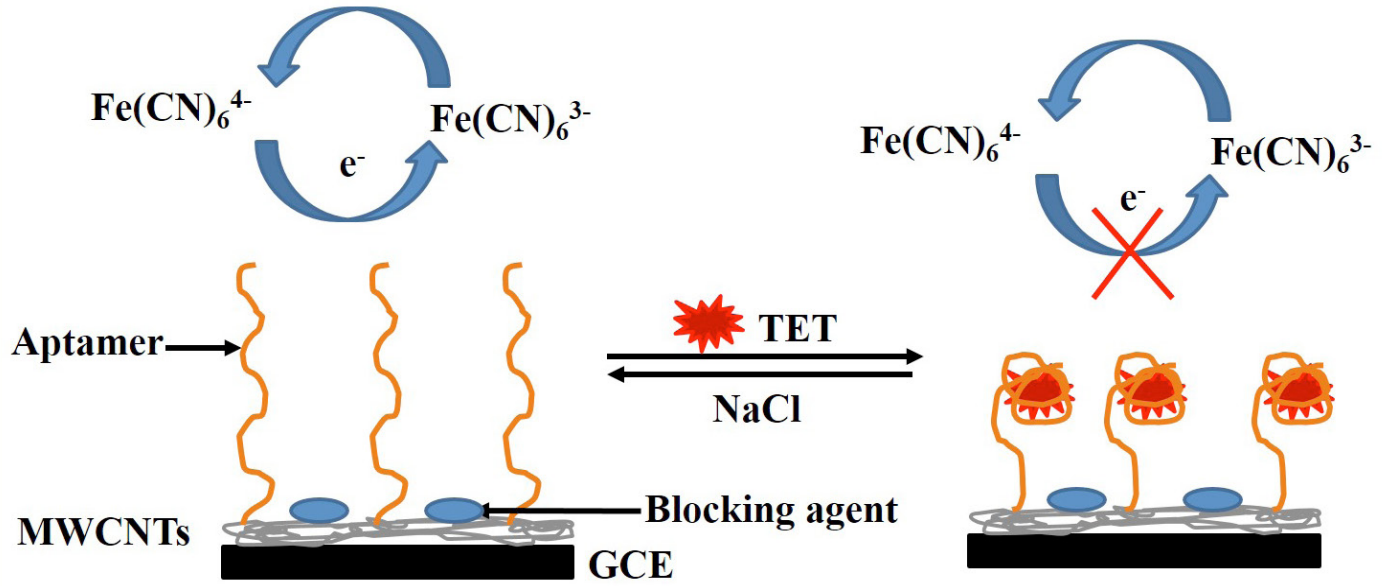

Figure 3: *Schematic of an electrochemical aptasensor for the detection of tetracycline using nanomaterial (Scheme illustration from Zhou et al., 2012)*

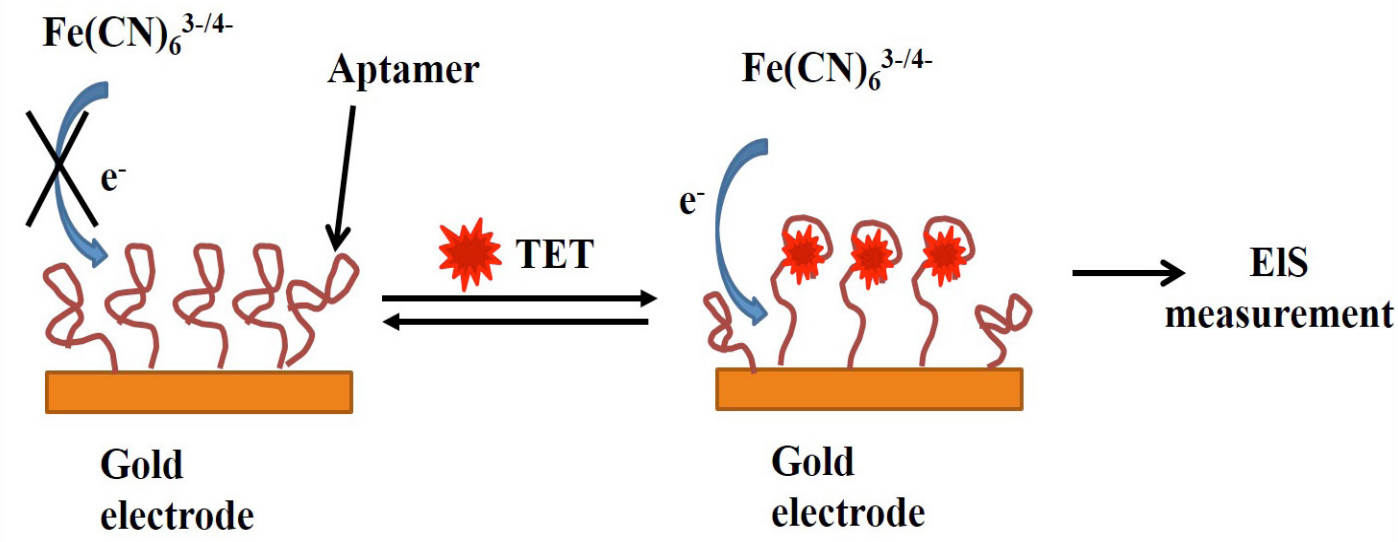

Figure 4: *Schematic of an electrochemical aptasensor for the detection of tetracycline (Scheme illustration from Chen et al., 2014)*

with a limit of detection 3.7 pg mL^{-1}. Guo et al. (2015) also demonstrated their aptasensors feasibility in milk samples and compared performance with the ELISA technique. The integration of nanomaterial improves the method performance in terms of sensitivity, stability and specificity. Very recently, a signal-on electrochemical DNA sensor combined with multiple recycling amplification has been reported for ultrasensitive detection of kanamycin (Wang et al., 2016). The multiple recycling amplification strategy used in the biosensor construction exhibited excellent sensitivity for kanamycin with a LOD of 1.3 fM.

One of the most popular classes of electrochemical biosensors is the impedimetric based sensing platform, where redox-active aptamers are immobilized onto a conducting surface to probe for electron transfer. On target binding, the aptamer folding into antiparallel quadruplex structure complexes that either shield or promote electron transfers between redox-coupled materials and electrode surface. The current state of electrochemical impedance spectroscopy (EIS) technology offers the advantages of improvement in disposability, portability and sensitivity of the developed method. Chen et al. (2014) reported the development of label-free electrochemical aptasensor specifically for the detection of tetracycline (TET) using anti-TET binding aptamer. For quantification of TET, the binding interaction between TET and its cognate aptamer was investigated using EIS. The obtained results suggested that the proposed aptasensor was highly sensitive provided the short incubation time and simpler development using gold surface (Figure 4). The aptasensor could sense TET concentrations as low to 1.0 ng mL^{-1} with an analysis time of 15 min.

Furthermore, this aptasensor shows good stability and reproducibility on performing real sample analysis in milk. Similarly, the comparison of two EIS-based aptasensor for detection of TET has been reported (Jahanbani and Benvidi, 2016). Recently, the EIS-based aptasensor has been demonstrated for detection of TET with recovery percentages of 88.1%–94.2% in spiked milk samples (Li et al., 2016).

In 2011, González-Fernández et al. (2011) demonstrated the development of EIS aptasensor (displacement assay) for detection of tobramycin in human serum. They used a competition between high affinity aptamer with free and bound forms of the analyte for signal measurement. The aptasensor was fabricated on the gold surface using thiol chemistry (Figure 5). The binding of anti-tobramycin aptamer to the target caused an increase in electron transfer resistance, due to negative charges of the phosphate groups present on the aptamers backbone. The target-aptamer complex was displaced the from electrode surface to form a complex with free tobramycin in solution, resulting in a reduction of negative charged leading to a decrease in resistance measured by the sensor. The researchers compared the performance of two functionally modified aptamer sequences. A dynamic range of 3.0 to 72.1μM with LOD of 1.8 μM was obtained in human serum. A label free all polymer-based biosensor for detection of ampicillin and kanamycin A has been reported (Dapra et al., 2013).

Very recently, an electrochemical aptasensor based on the arch shape structure of aptamer-conjugate complimentary strand and exonuclease has been demonstrated for sensitive detection of the streptomycin (Mohammed Danesh et al., 2016). The exonuclease acts as a digestive enzyme, which specifically degrades the ssDNA from its 3′-terminus end. The addition of streptomycin induces conformational changes between aptamer/streptomycin conjugate and release complementary strand (CS, act as ssDNA) from the aptamer. The addition of exonuclease causes the degradation of CS and results in the generation of the electrochemical signal. The performance of this aptasensor has been successfully demonstrated in milk and human serum samples. Guo et al. (2015) also reported a nanomaterial-based electrochemical aptasensor for detection of tetracycline in milk samples based on DPV signal generation. A polymerase catalyzed target recycling amplification with strand displacement aptasensor platform has been reported for ultrasensitive detection of antibiotics at very low level (Wang et al., 2015). The signal amplification technique results in the 100 -fold improvement in the detection limit for ampicillin. Similarly, a group of researchers also reported a dual recycling

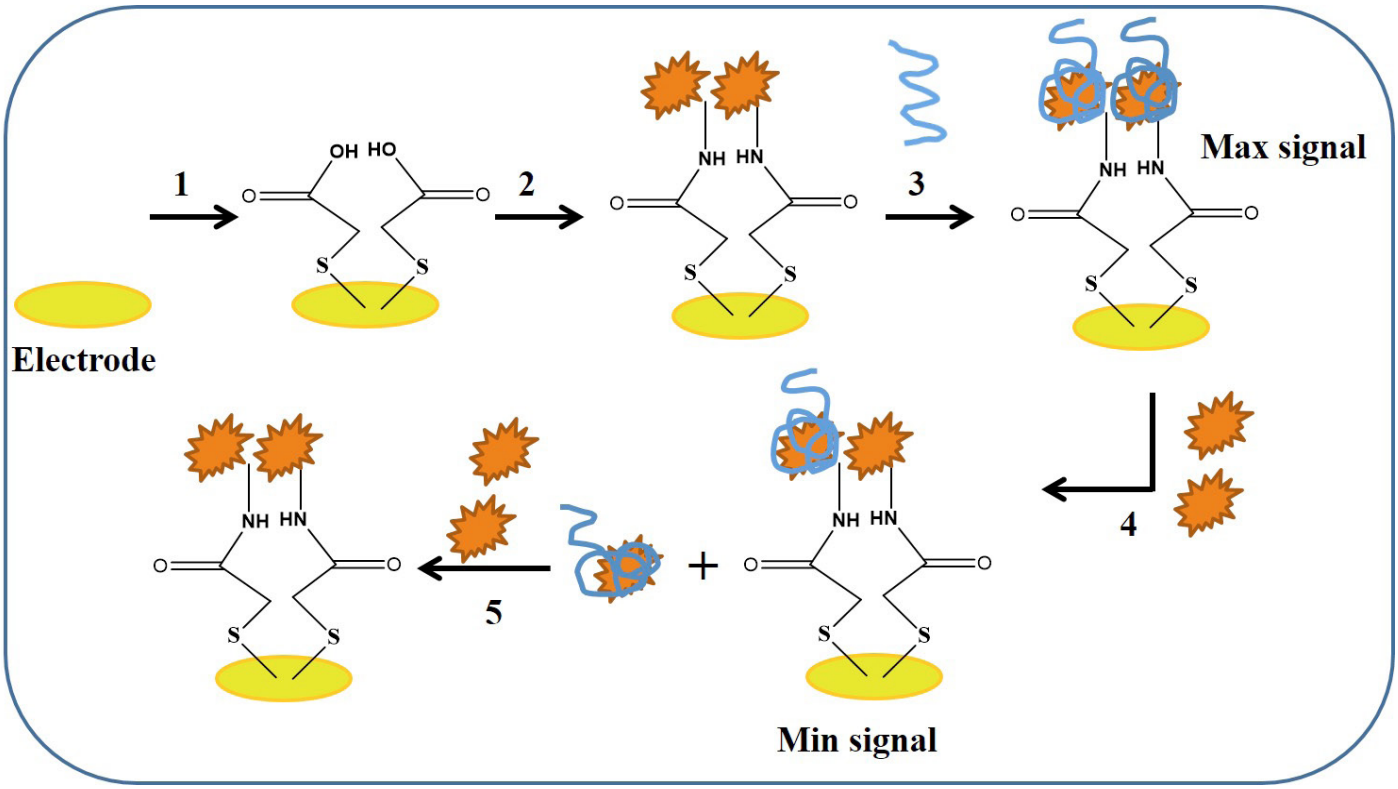

Figure 5: *Schematic representation of an aptamer displacement assay:* **A)** *Incubation;* **B)** *Sensing phase regeneration;* **C)** *Saturation of the sensing phase with the aptamer (Scheme illustration from González-Fernández et al., 2011)*

amplification strategy for ampicillin detection (Wang et al., 2016). Another group of researchers reported, the development of a square wave voltammetry-based electrochemical aptasensor for the detection of the oxytetracycline and chloramphenicol (Zheng et al., 2013; Yan et al., 2016). In the recent years, the development of nanoparticles mediated detection system has emerge out as promising potential, where nanoparticles either enhanced the electron transfer or loading capacity of biorecognition element. A 'turn-off/turn-on' gold nanoparticles (GNPs) based aptasensing approach integrated with the intrinsic peroxidase-like activity of GNPs with the high affinity and specificity of a ssDNA aptamer has been presented for the ultrafast and sensitive detection of small molecule such as kanamycin (Sharma et al., 2014). A novel sandwich-type electrochemical aptasensor has reported for detection of oxytetracycline (OTC) (Liu et al., 2016). The aptasensor was based on graphene-three dimensional nanostructure gold nanocomposite (GR-3D Au), which used an aptamer-AuNPs-horseradish peroxidase (aptamer-AuNPs-HRP) nanoprobes for signal amplification. This aptasensor showed a limit of detection of 4.98×10^{-10} g L^{-1} with a potential of successfully employed for food safety and clinical diagnosis.

Conclusions and Future Prospective

In conclusion, antibiotic resistance has emerged as a global threat and a future challenge. The problem of antibiotic contamination and drug resistance is not limited to one country, but affects the whole world due to globalization. Various conventional techniques for the analysis of antibiotic residues in agricultural, food and clinical therapy have been reported. With high affinity and selectivity, aptamers have been reported for different antibiotic. The wide distribution of antibiotics for prophylaxis of disease in humans and the environment or staple foods posed requirements for regular monitoring and screening. Requisite analytical methods should be able to detect antibiotic residues as much as low as the recommended levels. Although very innovative, many of the reported and described conventional and bioassay methods are impractical for field analysis, mainly when resources and highly skilled technicians are required, but not available in the field. Electrochemical signal generation-based aptasensors offers the advantages of label-free, disposable, fast, on-site analysis, miniaturization and portability. Therefore, the focus has been moved towards the development of a simple, robust, label-free, rapid yet sensitive tools based on electrochemical responses appear to be offer, for the near future, versatile, portable and sensitive field use devices for detection of antibiotics residue contamination in various types of different real samples.

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Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contribution

Atul Sharma (A.S.) performed the survey of literature and wrote the manuscript. Aruna Chandra Singh (A.C.S.) also contributed in the literature survey and scheme designing. Gautam Bacher (G.B.) contributed in the literature survey and manuscript formatting. Sunil Bhand (S.B.) was the investigator of this review and designed the manuscript.

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