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# Nanomaterial-based fluorescent biosensors for veterinary drug detection in foods

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## Abstract

Veterinary drugs have been widely used in the food industry. Their residues in food products need to be tightly regulated to ensure food safety. In particular, some veterinary drugs are still used illegally, although they have already been banned. Nanomaterials are playing an increasingly important role in analytical chemistry due to their unique properties. Compared to traditional organic dyes and colloidal gold nanoparticle labels, fluorescent nanomaterials appear particularly attractive for the detection of veterinary drug residues. This review summarizes recent advancements of fluorescent biosensors using nanomaterials for the detection of veterinary drug residues in foods. The useful properties of each type of fluorescent nanomaterial are first discussed such as large Stokes shifts, long emission lifetime, and high quantum yields, which are useful for detection in food-related sample matrix. The following target recognition molecules are then reviewed individually including antibodies, aptamers, molecularly imprinted polymers, and metal ion coordination based ligands. Representative bioconjugation and assay methods are discussed for each recognition mechanism. Finally, a few future research directions are outlined in the last section of this review.

**Keywords:** Antibodies, Aptamers, Biosensors, Fluorescence, Nanoparticles

## 1. Introduction

Food contamination is a major threat to human health, and recall of spoiled food can lead to huge economic losses in the food industry [1]. For various reasons, many illicit components exist in food products. Some are intentionally added, such as melamine for artificially boosting the nitrogen content that is used to reflect the protein content. Others are produced by food spoilage, or inadvertent exposure to trace toxicants, which may also increase the potential of food contamination [2].

Veterinary drug residues exist mainly in animal-derived products such as meat, milk, and eggs. To ensure effective feeding and fast growth of animals, and prevention and treatment of animal diseases, various veterinary drugs were used, such as hormones, growth promoters, and antibiotics [3].

Therefore, potential drug residues in animal-derived foods may cause adverse health effects such as cancer, disrupted hormone functions, and drug resistance [4,5]. For example, chloramphenicol can cause leukemia [6]; penicillin may cause the rash outbreaks [3]; while sulfanilamide and tetracycline are known for allergic reactions in susceptible bodies [7,8]. Therefore, the detection of veterinary drugs is of critical importance.

Technologies for detecting veterinary drug residues in foods are an important defense line for regulating market behavior and ensuring food safety. Detection of veterinary drugs is mainly needed by market regulation in animal farms, food manufacturing facilities, restaurants, retail markets, and wholesale markets [3], and it plays a crucial role in establishing public scientific health guidelines [9].

Many conventional methods were established for the detection of food contaminants. These methods

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include thin-layer chromatography (TLC) [10], high-performance liquid chromatography (HPLC) [11], liquid chromatography-mass spectrometry (LC-MS) [12], gas chromatography combined with mass spectrometry (GC-MS) [13], time-of-flight mass spectrometry (TOF-MS) [14] and capillary electrophoresis (CE) [15]. Nevertheless, the above methods have some inherent drawbacks, such as time-consuming and needing expensive instruments [9,16]. These complicated and laborious methods were difficult to meet the needs of fast and on-site screening of massive samples.

Therefore, rapid, portable, and effective detection methods are critical to ensure food safety. The complexity of food sample matrix and difficulties associated with tracking food contaminants pose additional challenges. Over the past decade, a large number of biosensors were reported as supplementary detection tools offering exciting alternatives to traditional methods for food contaminant detection [17]. Antibodies and aptamers are the main sensing molecules to develop biosensors.

In recent years, nanomaterials have gained popularity in biosensors and become a key component in the detection of microbes [18], environmental pollutants [19], toxins [20], pesticides [21], and veterinary drug residues [9], because of their excellent optical, magnetic and adsorption properties [22]. The published reviews on nanomaterial-based biosensors for veterinary drug detection mainly focus on optical and electrochemical detection of antibiotics [16] or electrochemical and electrochemiluminescent sensors [4,9].

Optical biosensors have a few advantages owing to their simplicity, specificity, and sensitivity. Fluorescence biosensors as the most exploited for food contaminants detection, due to its high sensitivity, wide dynamic range and versatile detection methods [22]. A diverse range of nanomaterial-based fluorophores are often brighter (higher fluorescence quantum yield), more stable, and sometimes have sharper emission peaks compared to organic fluorophores, allowing highly sensitive and multiplexed detection [23]. In addition, nanomaterials were used as immobilization platforms due to their unique features, such as a high specific surface area, and surfaces that can be readily modified with ligands. This review focuses on fluorescent biosensors based on nanomaterials for detecting veterinary drugs. We aim to critically review the main sensor design ideas by considering the food sample matrix.

## 2. Monitoring of veterinary drugs

### 2.1. Characteristics of veterinary drug

Veterinary drugs can be divided into the following types: antibiotics, synthetic antibacterials, antiparasitic drugs, and growth-promoting agents [24]. The chemical structures of some representative veterinary drugs are illustrated in Fig. 1. Although acute poisoning caused by veterinary drug residues in foods is extremely unlikely, low-level and long-term uptake still can produce various chronic and accumulative toxicity [25]. The harm of intake of veterinary drug residues is often unnoticed until a substantial and irreversible symptom is produced. Thus, stringent maximum residue limits (MRL) for veterinary drugs in animal-derived foods have been established by various regulatory authorities, including the European Union (EU) [26], China [27], USA [28], and Japan [29]. For instance, the MRL of chloramphenicol considered by China and the EU in milk is  $0.3 \mu\text{g L}^{-1}$  [26,27].

### 2.2. Characteristics of veterinary drug residues

Veterinary drug residues refer to the drug primitives, metabolites and impurities stored in cells, tissues or organs after giving the drugs to animals [30]. The use of banned and unqualified veterinary drugs, change of administration route, and non-compliance with the withdrawal period would cause veterinary drug residues [31]. The detection of veterinary drug residues is challenging for the following reasons. (1) Sensor signals have to be strong enough and the binding of the target has to be sufficiently specific when applied in a food sample matrix such as meat, milk, and eggs. (2) The detection needs to be sensitive enough to meet tight regulatory guidelines because the veterinary drug residues are generally at the level of part per billion (ppb).

### 2.3. Food sample pretreatment

As one of the key steps in veterinary drug residue analysis, food sample pretreatment directly affects the efficiency and accuracy of detection, and complicated pretreatment steps may increase the detection time [32]. Sample pretreatment starts from sampling to chemical analysis, including sample homogenization, reduction, sieving, centrifugation, filtration, antiseptic, and inhibition of degradation [33]. The complexity of food matrices frequently troubles analysis. In designing sample treatment

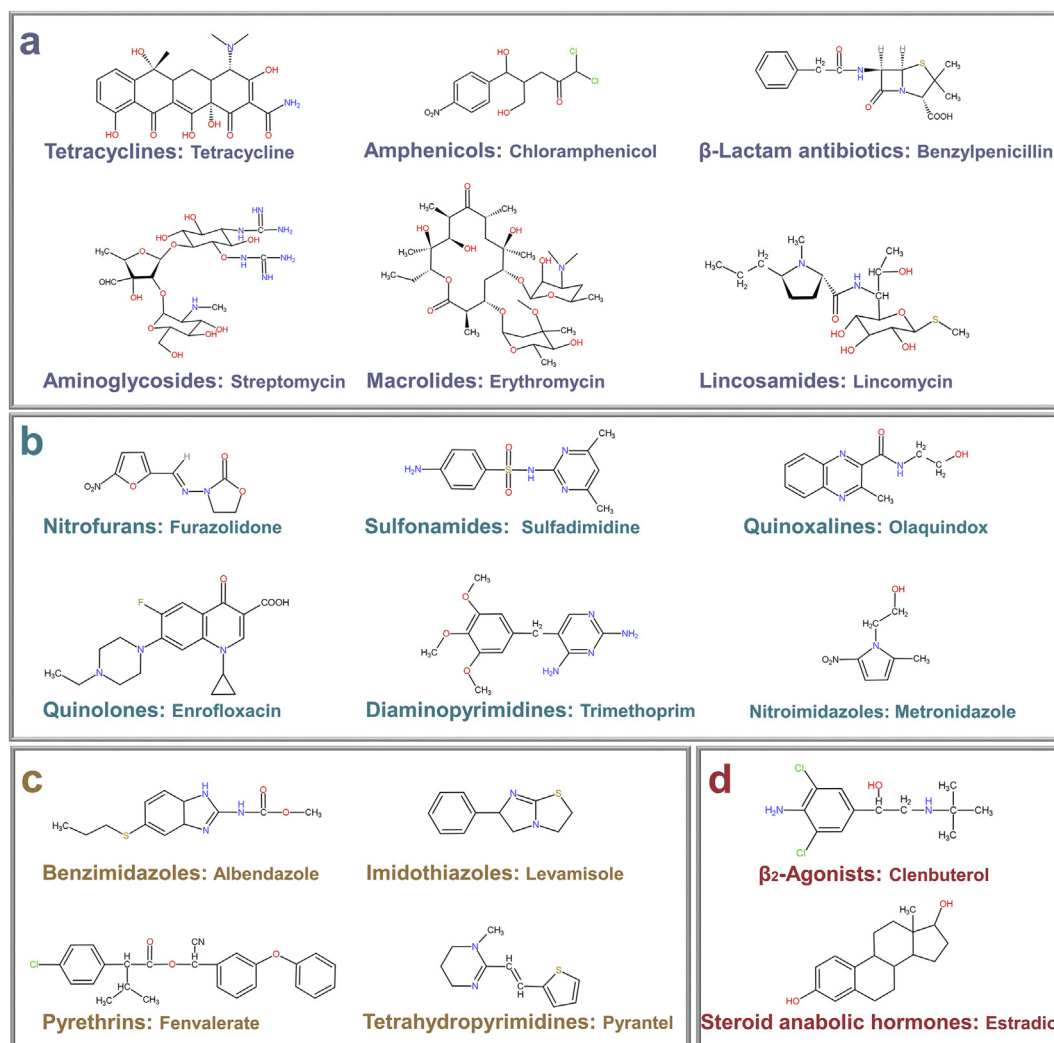


Fig. 1. Structures of some common veterinary drugs: (a) antibiotics, (b) synthetic antibacterials, (c) antiparasitic drugs, (d) growth-promoting agents.

methods, it is necessary to consider the physical and chemical properties of each sample type, and the state of the component to be tested. Some common pretreatment methods are summarized in Table 1 [34].

### 3. Fluorescent nanoparticles

Most of fluorescent nanoparticles used in the current detection of veterinary drug residues mainly

include fluorescent microspheres, quantum dots, carbon dots, and metal nanoclusters. The design of fluorescent nanoparticles includes: (1) encapsulating fluorescent materials inside microspheres (Fig. 2a1, Fig. 2a2) [35,36]; (2) decorating fluorescent materials on the surface of microspheres (Fig. 2a3) [37]; (3) decorating an inorganic layer on the surface of quantum dots forming a core–shell structure or combing an organic functional layer (Fig. 2b) [38]; and (4) using fluorescent metal nanoclusters (Fig. 2c)

Table 1. Common food sample pretreatment for biosensors.

Methods	Advantages	Disadvantages
Nitrogen blowing	(1) Concentration without oxygen (2) Time-saving (3) Easy to operate and control	Suitable only for small volume or size of samples
Immunoaffinity chromatography (IAC)	High selectivity and stability	Complicated preparation of antibodies
Solid-phase extraction (SPE)	(1) Time-saving (2) Achieving the trace separation (3) High selectivity and reproducibility	(1) High cost of SPE columns (2) Need professional operators
Rotary evaporation	(1) Flash distillation (2) Suitable for bulk sample concentration	Boiling may occur, resulting in a loss of samples.

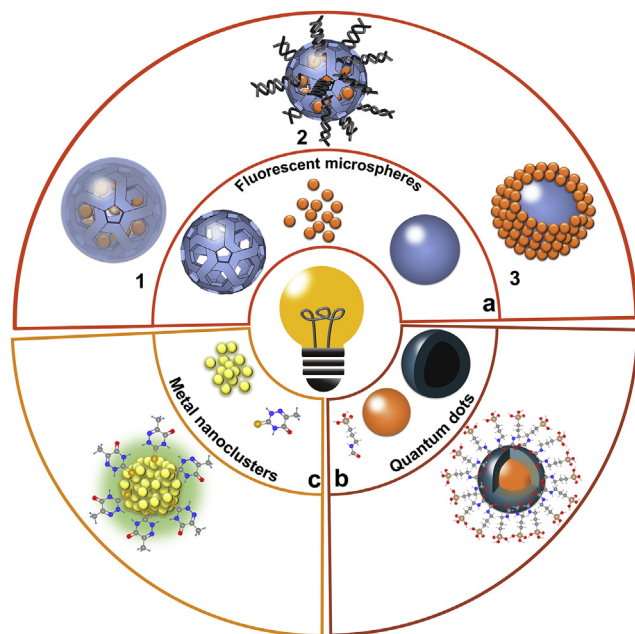


Fig. 2. Schematic illustration of various fluorescent nanoparticles for the detection of veterinary drugs: (a) fluorescent microspheres; (b) quantum dots; and (c) metal nanoclusters.

[39]. Some promising materials to prepare fluorescent microspheres are organic dyes such as fluorescein isothiocyanate and rhodamine B [36] due to their high quantum yields and low cost. However, conventional organic dyes suffer from photobleaching and spectral overlapping [37]. In contrast, inorganic fluorescent materials, such as quantum dots, may overcome these challenges due to their excellent photostability, narrow and tunable emission spectra, and single excitation wavelength for different emitters [37].

### 3.1. Fluorescent microspheres

#### 3.1.1. Fluorescent microspheres labelled with organic dyes

Fluorescence microspheres were manufactured by high-quality polystyrene and loaded with a variety of dyes (Fig. 2a1) [37]. The surface of fluorescent microspheres was easily functionalized (e.g. terminated by carboxylate or amine groups) to bind to targets. Due to strong  $\pi$ - $\pi$  interactions in the aggregated state, a high concentration of traditional organic dyes may sometimes suffer from aggregation-induced quenching [40]. Therefore, materials based on aggregation-induced emission (AIE) became a new generation of fluorescent labels because of the superior brightness in the aggregate state, larger Stokes shift, and higher relative quantum yield [41,42]. Compared to individual

fluorophores, these fluorescent microspheres drastically amplify detection signals, which are particularly attractive for detection in food samples to overcome the background fluorescence.

#### 3.1.2. Fluorescent microspheres labelled with lanthanide complexes

Lanthanide (e.g., Tb(III) and Eu(III)) labelled nanomaterials have unique characteristics long-lifetime emissions (up to 1 ms) and large Stokes shifts (over 150 nm) [43]. Therefore, interference from background fluorescence could be significantly reduced by means of a time delay that can be realized by time-gated luminescence (Fig. 3a) [44]. Lanthanide-doped up-conversion nanoparticles (UCNPs) demonstrated promising applications for sensitive detection of antibiotics [45]. With near-IR light of 980 nm for excitation, most background fluorophores cannot be excited while the UCNPs were efficiently excited, thus showing excellent optical features such as low phototoxicity and low autofluorescence background (Fig. 3b) [46].

#### 3.1.3. Fluorescent nanosilica

Silica is a popular material for dye containment, and it has several advantages compared to polymer-based materials due to its high stability, low cost, and easy to synthesis. Fluorescent nanosilica are obtained by encapsulating fluorescent materials during sol-gel synthesis of silica nanoparticles. Silica can protect dyes such as ruthenium(II) complexes from oxidation and improve the photochemical stability and fluorescence intensity [47]. As a transition metal-based luminophore,  $\text{Ru}(\text{phen})_3^{2+}$  has potential applications in biosensors due to a high emission quantum yield, large Stokes shift, and long fluorescence lifetime [48]. Dyes can also be adsorbed into mesoporous silica nanoparticles (MSNs) and gated by the structure switching of aptamers after binding targets (Fig. 2a2) [49,50].

### 3.2. Quantum dots

Fluorescent semiconductor nanoparticles known as quantum dots (QDs) have received widespread attention with the promise to replace traditional organic dyes owing to their unique optical properties such as narrow emission bands and brightness [4]. They were used in immunoassays [51] and aptasensors [52] for veterinary drug detection. Most QDs have a core-shell structure (passivating QDs with an inorganic layer to improve its quantum yield and narrow the emission spectrum), such as CdSe QDs with a ZnS shell (ZnCdSe/ZnS) (Fig. 2b) [38].



Table 2. Overview of fluorescent immunosensing platforms using nanomaterials for veterinary drug detection.

Type	Analyte	Sample	Labelling Methods	Detection limit	Ref.
FLISA	Quinolones	Milk	Carboxylate-modified QD655 labelled secondary antibody	0.18 $\mu\text{g L}^{-1}$	[51]
	Sulfonamides		Avidin coated QD605-biotinylated primary antibody	0.17 $\mu\text{g L}^{-1}$	
	Melamine			7.5 $\mu\text{g L}^{-1}$	
FLISA	Sulfamethazine	Chicken muscle tissue	Carboxylate-modified QD655 labelled secondary antibody	1.0 $\mu\text{g L}^{-1}$	[64]
FLISA	Enrofloxacin	Chicken muscle tissue	Carboxylate-modified QD655 labelled secondary antibody	2.5 $\mu\text{g L}^{-1}$	[65]
FLISA	Tylosin	Edible animal tissues	Carboxylate-modified QD655 labelled secondary antibody	0.02 $\mu\text{g L}^{-1}$	[66]
	Tilmicosin			0.04 $\mu\text{g kg}^{-1}$	
FIA	Norfloxacin	Animal-derived foods	Carboxyl-modified $\text{NaYF}_4:\text{Yb,Tm}$ UNCPs labelled anti-target MAb	0.01 $\mu\text{g L}^{-1}$	[67]
FIA	Sulfaquinolaxaline	Animal-derived foods	Carboxyl-modified $\text{NaYF}_4:\text{Yb,Tm}$ UNCPs labelled anti-target MAb	0.5 $\mu\text{g kg}^{-1}$	[68]
FICA	Kanamycin	Milk	BHHCT-Eu <sup>3+</sup> @ SiO <sub>2</sub> labelled anti-target MAb (glutaraldehyde crosslinking)	0.85 $\mu\text{g L}^{-1}$	[35]
FICA	Sulfamethazine	Honey	Carboxyl groups on AIEFM labelled anti-target MAb	0.028 $\mu\text{g L}^{-1}$	[41]
FICA	Enrofloxacin	Animal tissue	Carboxylate-modified ZnCdSe/ZnS QDs labelled anti-target MAb	5 $\mu\text{g kg}^{-1}$	[69]
FICA	Ractopamine	Swine urine	Carboxylate-modified dyed polymer microsphere labelled anti-target MAb	10 $\mu\text{g L}^{-1}$	[70]
			Carboxylate-modified time-resolved fluorescent nanobeads labelled anti-target PAb	7.2 ng L <sup>-1</sup>	
			Fluorescent submicrospheres labelled anti-target PAb	0.0147 $\mu\text{g L}^{-1}$	
FICA	Clenbuterol	Swine urine	Carboxylate-modified QDs labelled anti-target PAb	0.0236 $\mu\text{g L}^{-1}$	[71]
		Carboxylate-modified Fluorescent magnetic nanobeads labelled anti-target MAb	0.22 $\mu\text{g L}^{-1}$		
FICA	Lincomycin	Milk, honey, beef	Carboxylate-modified FluoSpheres® labelled anti-target MAb	0.69 $\mu\text{g L}^{-1}$	[72]
FICA	Sulfamethazine	Milk	Carboxylate-modified FluoSpheres® labelled anti-target MAb	0.11 $\mu\text{g L}^{-1}$	[73]
FICA	Tilmicosin	Milk	Carboxylate-modified FluoSpheres® labelled anti-target MAb	0.19 $\mu\text{g L}^{-1}$	[74]
FICA	Enrofloxacin	Chicken meat	Ru(phen) <sub>3</sub> <sup>2+</sup> -doped FN labelled anti-target MAb (EDC crosslinking)	0.02 $\mu\text{g L}^{-1}$	[75]
FICA	Oxytetracycline	Edible animal tissues	QDs labelled anti-target MAb (EDC crosslinking)	0.44 $\mu\text{g L}^{-1}$	[76]
FICA	Gentamicin	Milk	Carboxylate-modified QDs labelled anti-target PAb	2 $\mu\text{g kg}^{-1}$	[77]
FICA	Ractopamine	Swine urine and muscle tissue	[FRET] Carboxylate-modified fluorescent polymer dots labelled BSA AuNPs labelled anti-target MAb act as quencher	0.16 $\mu\text{g L}^{-1}$	[78]
FICA	Tetracycline antibiotics	Milk	[FRET] Carboxylate-modified ZnCdSe/ZnS QDs labelled OVA	20 $\mu\text{g L}^{-1}$	[79]
		Animal muscle tissue	AuNPs labelled anti-target MAb act as quencher	40 $\mu\text{g kg}^{-1}$	
MLFIA	Clenbuterol	Swine urine	Carboxyl groups on Arg/ATT/AuNCs labelled anti-target MAb	0.25 $\mu\text{g L}^{-1}$	[39]
FTIACT	Sulfonamide	Milk	Liposome-encapsulated QDs labelled with hapten-carried protein conjugates via N-succinimidyl 3-(2-pyridyldithio) propionate	0.13 $\mu\text{g L}^{-1}$	[55]
	Quinolone			0.062 $\mu\text{g L}^{-1}$	
FICA	Macrolides	Raw milk	Carboxylate-Modified FluoSpheres® labelled antibody	0.13 $\mu\text{g L}^{-1}$	[80]

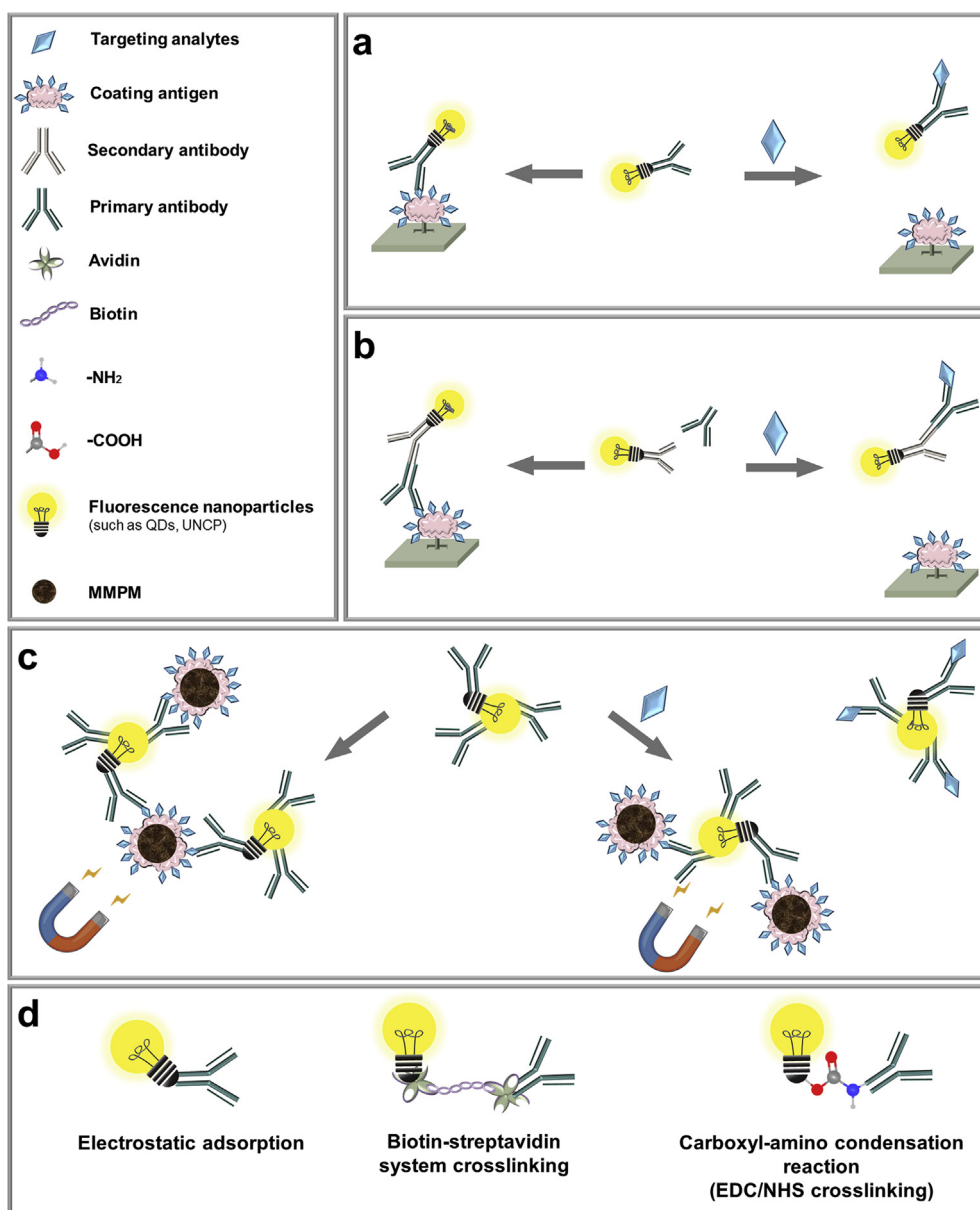


Fig. 4. Schematic illustration of fluorescence-linked immunoassays for the detection of veterinary drugs: (a) direct competition; (b) indirect competition; and (c) combined with immunomagnetic separation. (d) Bioconjugation methods between antibodies and fluorescent nanoparticles.

(Fig. 4). Fluorescent nanoparticles labelling of antibodies was mainly achieved by means of biotin-streptavidin binding, electrostatic adsorption, and the amino-carboxyl condensation reaction (Fig. 4d) [51,64,68]. The amino-carboxyl condensation reaction is typically facilitated by EDC/NHS coupling (EDC: 1-ethyl-3-[3(dimethylamino)propyl]carbodiimide; NHS: N-hydroxysuccinimide) [68].

#### 4.1.1. Direct competition

As depicted in Fig. 4a, a fluorescence-linked immunosorbent assay (FLISA) was developed using primary antibodies modified with fluorescent

nanoparticles. In the absence of targets, the labelled primary antibodies bound to the coating antigens on a microplate. In the presence of targets, the targets and the coating antigens competed to bind with the labelled primary antibodies, and the labelled primary antibodies remained in solution were washed away, leading to a lower fluorescence on the microplate (Fig. 4a).

#### 4.1.2. Indirect competition

As described in Fig. 4b, indirect competition FLISA used secondary antibodies labelled with fluorescent nanoparticles to bind to primary



antibodies. Compared to the direct competitive assay, the labelled secondary antibody was added after adding primary antibodies. QDs were usually linked to secondary antibodies via the biotin-streptavidin conjugation [51] or the amino-carboxyl condensation reaction [64–66]. In particular, the unique binding characteristics of biotin/avidin (avidin as a bridge to the secondary antibody) decreased the adverse effect on the cross-reactivity of primary antibodies with directly conjugated QD. A FLISA was developed for the simultaneous determination of sulfonamides, quinolones, and melamine in milk [51].

#### 4.1.3. Immunomagnetic separation

FIA can also be combined with magnetic separation to further improve sensitivity (Fig. 4c). In the absence of targets, primary antibodies labelled with fluorescent nanoparticles could bind coating antigens (immobilized on magnetic polystyrene microspheres), and a strong fluorescence is detected in the system. In the presence of targets, targets bound to the labelled primary antibodies and are removed via magnetic separation, thus decreasing the fluorescence. The strategy was employed to detect sulfaquinoxaline in animal-derived foods, with a LOD of  $0.5 \mu\text{g kg}^{-1}$  [68].

### 4.2. Fluorescent immunochromatographic assay (FICA)

#### 4.2.1. Traditional fluorescent lateral flow immunoassay

Typical immunoassays require multiple washing and incubation steps. Although highly sensitive, it is difficult to apply these methods outside a lab. Immunochromatographic assay was developed to solve this problem by integrating the assays on a simple flow device, and the best-known example is the pregnancy test strips [81]. Fluorescent immunochromatographic assay (FICA) was also developed to detect veterinary drugs in food samples with a competitive immunoassay principle.

As shown in Fig. 5a, coating antigens were pinned to the test line of the strip to bind primary antibodies labelled with fluorescent nanomaterials, and secondary antibodies were pinned to the control line to capture the unbound primary antibodies (a positive control line indicates that the test result is valid). Without a target, the labelled primary antibodies can bind with the antigens coated on the test line, and the fluorescence is strongest. With the increase of target concentration, more targets bind to the labelled primary antibodies to decrease the signal.

A study compared FICAs based on anti-target polyclonal antibody labelled with QDs, fluorescent submicrospheres and time-resolved fluorescent nanobeads (TRFNs) for ractopamine detection in swine urine, with the LODs of  $23.6 \text{ ng L}^{-1}$ ,  $14.7 \text{ ng L}^{-1}$ ,  $7.2 \text{ ng L}^{-1}$ , respectively [70]. With a  $200 \mu\text{s}$  delay time in fluorescence measurement, TRFN-FICA had the lowest LOD, shortest detection time and widest linear range. Therefore, the above three FICAs are also suitable for the detection of veterinary drug residues in food samples, and the use of TRFNs recommended. FICA is well suited for the competitive format and has a broader prospect for veterinary drug detection in practical application with the obvious advantages (time-saving, easy operation, high sensitivity and specificity).

#### 4.2.2. Combined with immunomagnetic separation

FICA can also combine with immunomagnetic separation (Fig. 5b). Instead of just using a fluorophore label, primary antibodies are labelled on a fluorescent magnetic nanobead (FMNB), which is also used for sample purification. The principle of the subsequent detection process is similar to Fig. 5a. A FICA combined with immunomagnetic separation was established to detect the clenbuterol residue in swine urine with the LOD of  $0.22 \text{ ng mL}^{-1}$  [71], four times lower than that of traditional colloidal gold-based ICA. The magnetic separation can minimize the effect of the sample matrix in veterinary drug detection to boost sensitivity and specificity.

#### 4.2.3. Multi-component analysis

FICAs can also be used to simultaneously analyze multiple components or more than one group of veterinary drugs, and these are called multiplex lateral flow immunoassays (MLFIA). As shown in Fig. 5c, two test lines (test line 1 and 2) are marked with coating antigens of two different targets. In the absence of targets, the coating antigens on the test line bind with the antibodies labelled with fluorescent nanoparticles and show the highest fluorescence intensity. In the presence of one or both targets, targets bind to antibodies labelled with fluorescent nanoparticles, and the fluorescence of the corresponding test line (test line 1 and/or test line 2) decrease because of the competitive binding. The fluorescence intensity ratio of the test lines to the control line is used to quantitatively measure binding and offset the inherent heterogeneity of MLFIA. A MLFIA was developed for the simultaneous detection of clenbuterol and ractopamine in swine urine [39].

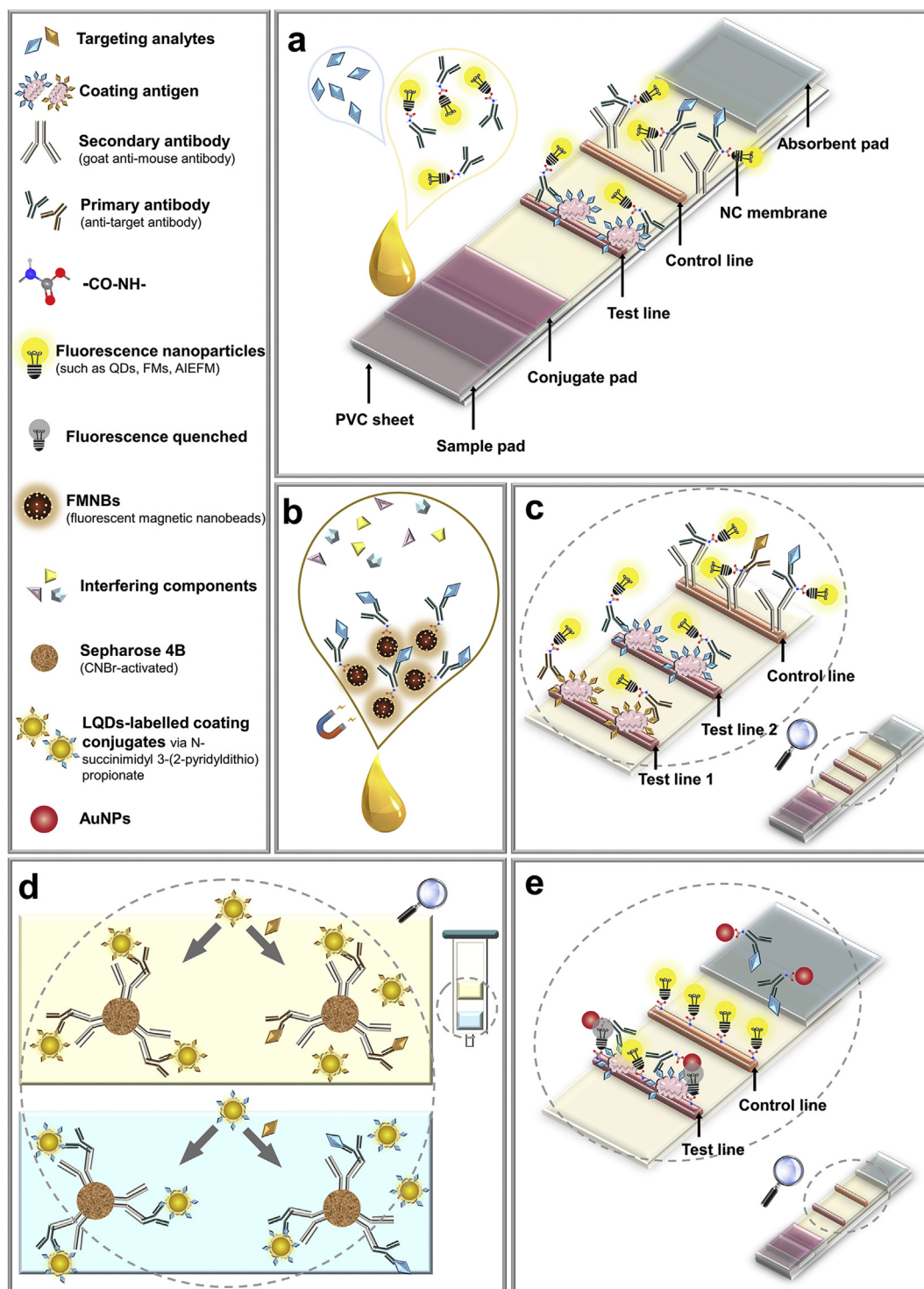


Fig. 5. Schematic illustration of fluorescent lateral flow immunoassay for veterinary drug detection: (a) traditional assay (figure created based on ref. [41]); (b) combined with immunomagnetic separation (figure created based on ref. [71]); (c) multiplex lateral flow immunoassay (figure created based on ref. [39]); (d) multiplex flow-through immunoaffinity chromatography test (figure created based on ref. [55]); (e) base on FRET (figure created based on ref. [79]).

A multiplex flow-through immunoaffinity chromatography test (FTIACT) is shown in Fig. 5d. In this design, the secondary antibody is labelled on sepharose 4B beads, and the primary antibodies are

attached to the secondary antibodies. The targets compete with liposome-encapsulated QDs-labelled targets to bind with the primary antibodies. A multiplex FTIACT was developed for the detection

Table 3. Overview of aptamer-based fluorescent biosensors using nanomaterials for veterinary drug detection.

Strategy	Analyte	Sample	Method	Detection limit	Ref.
Nanomaterials as fluorescence quenchers	Oxytetracycline	Honey, water	FRET from thiol-Aptamer modified AgNCs to target.	0.046 $\mu\text{g L}^{-1}$	[61]
	Sulfadimethoxine	Milk	FRET from Aptamer-FAM to CPNBs.	10 $\mu\text{g L}^{-1}$	[84]
	Oxytetracycline	Lake water	FRET from Aptamer-FAM to rGO.	4.6 $\mu\text{g L}^{-1}$	[85]
	Tetracycline	Milk	FRET from Aptamer-VS <sub>2</sub> QDs to MoS <sub>2</sub> nanosheets. (amino-carboxyl condensation reaction between VS <sub>2</sub> QDs and aptamer)	0.06 $\mu\text{g L}^{-1}$	[86]
	Tetracycline	Animal-derived food	FRET from Aptamer-N-GQDs to CoOOH. (amino-carboxyl condensation reaction between N-GQDs and aptamer)	0.95 $\mu\text{g L}^{-1}$	[87]
	Oxytetracycline	Tap water, river	FRET from Aptamer-FAM to GO hydrogel.	25 $\mu\text{g L}^{-1}$	[88]
	Kanamycin	Milk, animal-derived food	FRET from Aptamer-CDs to MoS <sub>2</sub> nanosheets. (amino-carboxyl condensation reaction between CDs and aptamer)	532.95 $\mu\text{g L}^{-1}$	[89]
	Kanamycin	Milk	FRET from Aptamer-FAM to rGO.	0.4845 $\text{ng L}^{-1}$	[90]
	Florfenicol	Milk	FRET from Aptamer-ATTO647N to GO.	2.06 $\mu\text{g L}^{-1}$	[91]
	Oxytetracycline	Spiked milk and tablets	FRET from oxytetracycline-Eu <sup>3+</sup> to AgNPs. (electrostatic adsorption between targets and Eu <sup>3+</sup> )	0.874 $\mu\text{g L}^{-1}$	[92]
Label-free sensors	Kanamycin A	Milk	FRET from Aptamer-FAM to AuNPs	0.145 $\mu\text{g L}^{-1}$	[93]
	Enrofloxacin	Milk	FRET from targets to GO.	1.33 $\mu\text{g L}^{-1}$	[94]
	Ofloxacin	Milk	FRET from Rhodamine B to AuNPs.	1.66 $\mu\text{g L}^{-1}$	[95]
	Kanamycin	Milk	IFE from CDs to AuNPs.	8.72 $\mu\text{g L}^{-1}$	[96]
Direct detection [Splitting aptamers]	Kanamycin	Human serum	One DNA split was labelled with CuS nanoparticle by amino-carboxyl condensation reaction, and the other was coupled with paramagnetic particles (PMPs) by biotin-streptavidin system crosslinking.	12.597 $\text{ng L}^{-1}$	[97]
Indirect competitive [Strand-displacement strategy]	Kanamycin	Milk	FRET from QDs-SSB to Aptamer-BHQ <sub>1</sub> (amino-carboxyl condensation reaction between QDs and SSB)	0.006 $\mu\text{g L}^{-1}$	[52]
	Oxytetracycline	Milk	FRET from FAM (labelled on CS) to GO.	4.6043 $\text{mg L}^{-1}$	[98]
	Oxytetracycline	Milk	LRET from NaYF <sub>4</sub> :Yb,Tm UCNPs to SYBR Green I. (amino-carboxyl condensation reaction between UCNPs and aptamer)	0.054 $\mu\text{g L}^{-1}$	[99]
	Kanamycin	Milk, serum	FRET from FAM (labelled on CS) to AuNPs.	0.156 $\mu\text{g L}^{-1}$	[100]

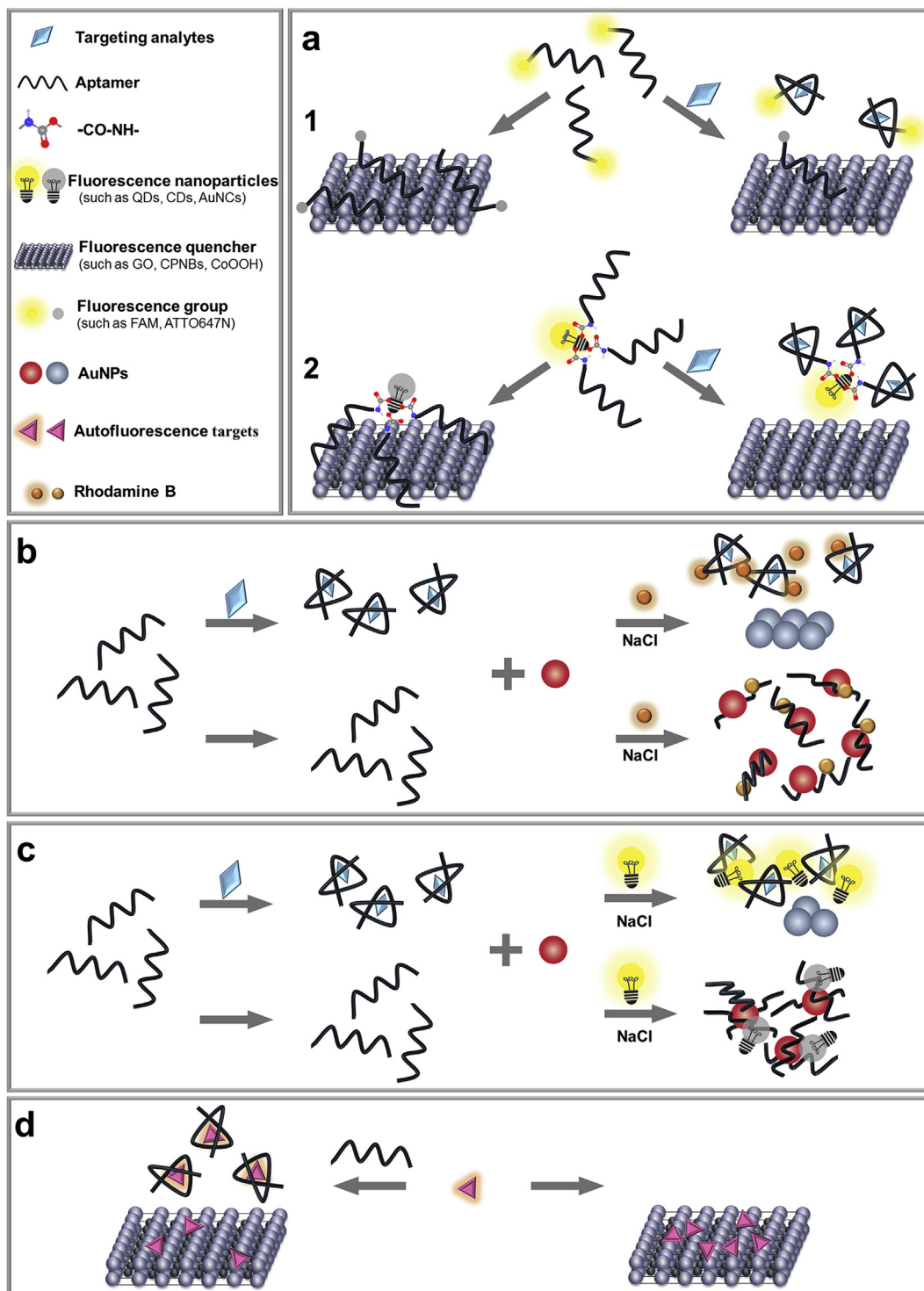


Fig. 6. Schematic illustration of assays based on nanomaterials as fluorescence quenchers and label-free sensors for the detection of veterinary drugs. (a) Labeled type (figure created based on ref. [84,86]). (b) Label-free assay base on FRET from rhodamine B to AuNPs (figure created based on ref. [95]). (c) Label-free assay base on IFE from CDs to AuNPs (figure created based on ref. [96]). (d) Label-free assay base on the autofluorescence of target (figure created based on ref. [94]).

of 14 sulfonamides and 13 quinolones residues in milk, with LODs of  $1 \text{ ng mL}^{-1}$  and  $0.5 \text{ ng mL}^{-1}$  for the sulfonamides and quinolones, respectively [55]. Multiplex FTIACT is promising for qualitative and quantitative detection of multiple veterinary drugs in food samples.

#### 4.2.4. Assays based on fluorescence resonance energy transfer

As shown in Fig. 5e, fluorescence resonance energy transfer (FRET) has also been used in FICA. In this example, the fluorescent polymer dots-labelled BSA was the fluorophore, and AuNP-labelled primary antibody (anti-target MAb) was the quencher. In the absence of targets, AuNP-labelled primary antibodies bound with the coating antigen on the test line, and the fluorescence of QDs (fixed on the test line) was quenched by AuNPs at this time. After adding targets, the FRET effect was reduced, and the fluorescence of the test line was high. This way, more fluorescence signal was produced in this competitive assay along with the increase in target concentration. This method allowed quantities analysis of ractopamine and tetracycline antibiotics in animal muscle tissues, with the LODs of  $0.16 \text{ ng mL}^{-1}$  [78] and  $40 \text{ } \mu\text{g kg}^{-1}$  [79], respectively.

## 5. Aptamer-based assays

Traditional immunoassays based on antigen–antibody recognition have a high production cost and antibodies may suffer from large batch-to-batch variation. Meanwhile, labelling of fluorophores is also complex and difficult to be achieved site-specific labeling. Nucleic acid aptamers are similar to antibodies in selective targets binding, and they are particularly suitable for the binding of

small molecular targets. Aptamers are isolated from an enriched nucleotide library by the SELEX (systematic evolution of ligands by exponential enrichment) method [82]. Aptamers have been widely used in environmental monitoring, food safety, and veterinary drug detection due to their simple and rapid synthesis, easy modification, low cost, high affinity, and high stability [83]. In recent years, aptamer-based fluorescent biosensors using nanomaterials for veterinary drug detection are shown in Table 3.

#### 5.1. Nanomaterials as fluorescence quenchers

As shown in Fig. 6a, adsorption of a fluorescently labelled aptamer on some nanomaterials can result in fluorescent quenching. In the presence of target analytes, the aptamer is desorbed to enhance the fluorescence signal. Common fluorescent quenchers including coordination polymer nanobelts (Fig. 6a1) [84], VS<sub>2</sub> QDs, molybdenum (IV) disulfide (MoS<sub>2</sub>) nanosheets (Fig. 6a2) [86], and other examples shown in Table 3. Sometimes, even the target molecules can act as fluorescent quenchers, such as oxytetracycline [61] and oxytetracycline-Eu<sup>3+</sup> [92].

It is worth noting that not all nanomaterials are equal. For example, it is very difficult to desorb aptamers from AuNPs due to an extremely strong adsorption affinity. Recent work showed that only less than 5% of adsorbed aptamers could be desorbed by target molecules, and even this small number of desorbed aptamers were likely due to nonspecific displacement instead of specific aptamer binding [82,101,102]. An aptasensor for the detection of kanamycin was developed by quenching of the FAM-labelled aptamer on AuNPs [93]. In

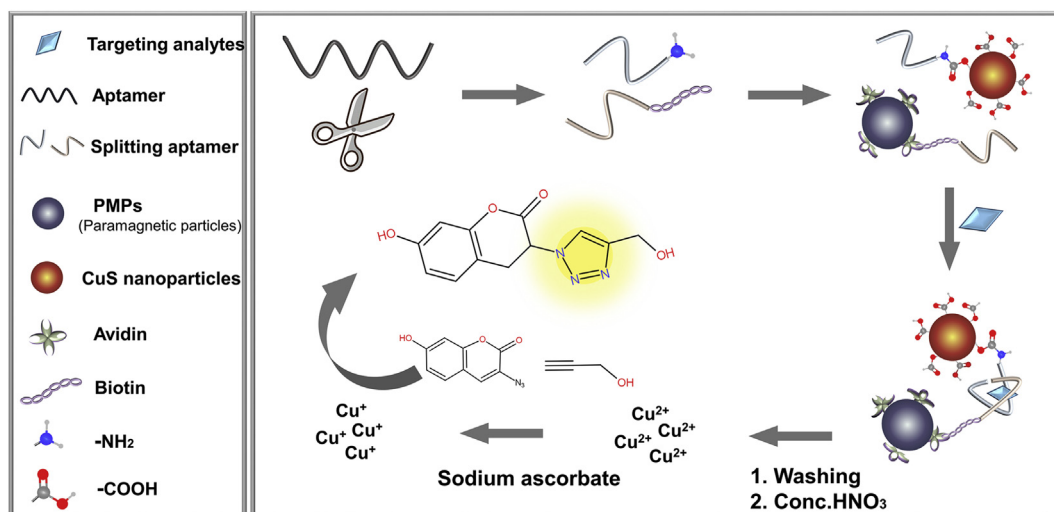


Fig. 7. Schematic illustration of assays based on the splitting aptamers for the detection of veterinary drugs (figure created based on ref. [97]).

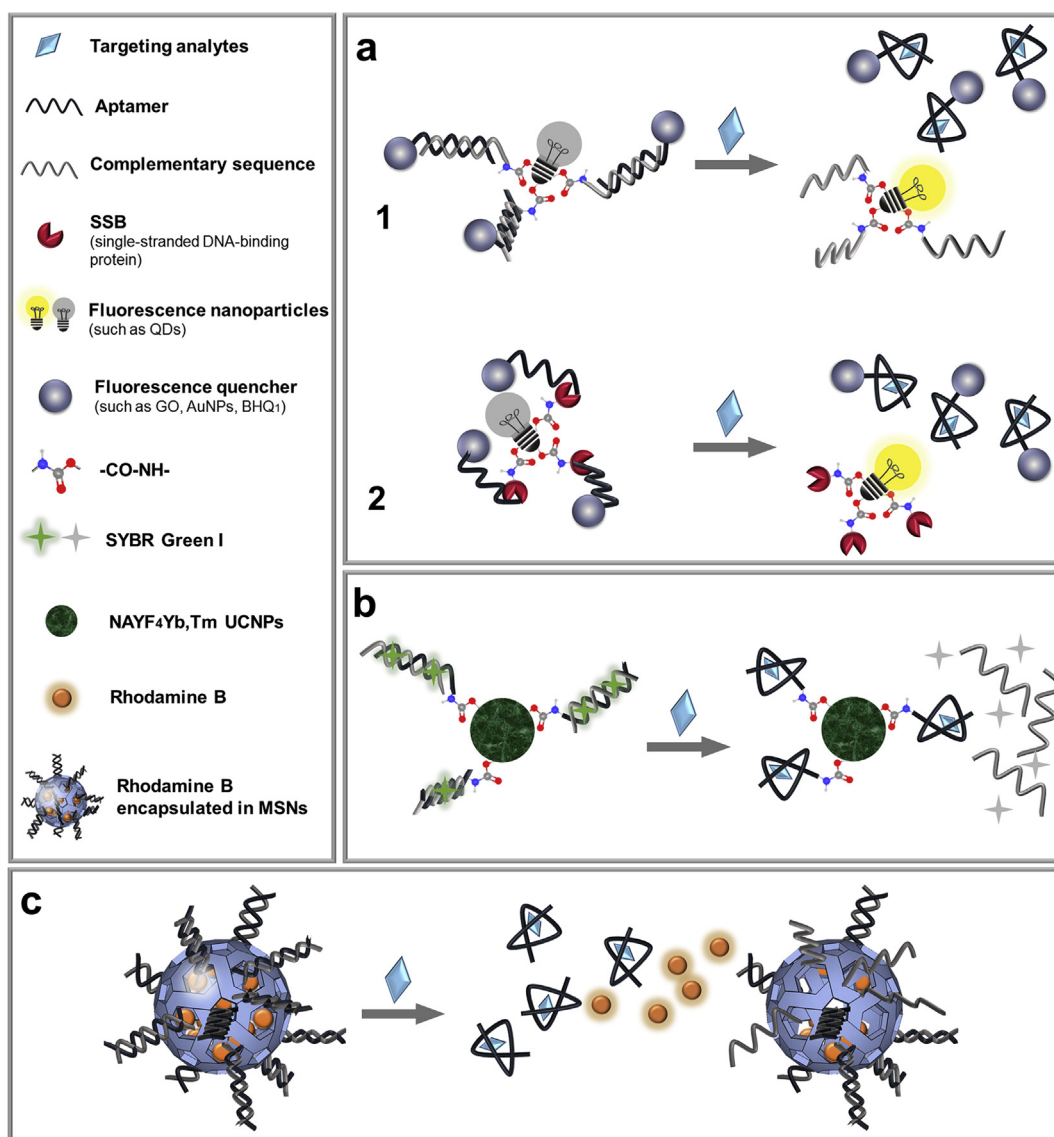


Fig. 8. Schematic illustration of assays based on the structure-switching aptamer strategies for the detection of veterinary drugs. (a) Assays based on FRET (figure created based on ref. [52,98]). (b) Assays based on LRET (figure created based on ref. [99]). (c) Assays based on gated release of dyes from MSNs (figure created based on ref. [36]).

this case, the saturated fluorescence enhancement was only about 25%. Although the authors did not show the total adsorbed DNA, based on our results, 10-fold or more enhancement can be readily achieved if all the adsorbed DNAs were desorbed (e.g. by adding KCN) [101,103].

## 5.2. Label-free sensors

Since label-free sensors do not require a covalent label, they are more cost-effective. As shown in Fig. 6b, a label-free strategy is described. In the absence of targets, AuNPs remain dispersed due to adsorbed aptamers in a high concentration of NaCl,

and the dispersed AuNPs could reduce the fluorescence intensity of Rhodamine B by the inner-filter effect. In the presence of targets, the aptamers were expected to bind to targets and thus could not be adsorbed on AuNPs. Therefore, the AuNPs were exposed and aggregated in the NaCl solution, resulting in a high fluorescence intensity [95]. Aptasensor for ofloxacin detection in milk was established with a LOD of  $1.66 \mu\text{g L}^{-1}$  [95].

Similarly, a strategy developed using the inner filter effect using fluorescence of CDs (Fig. 6c). An aptasensor was developed for kanamycin detection in milk with a LOD as low as 18 nM [96]. It needs to be noted that some targets (such as kanamycin) are

Table 4. Overview of target recognition based on molecularly imprinted polymers or metal coordination using nanomaterials for veterinary drug detection.

Type	Analyte	Sample	Fluorescent nanoparticles	Method	Detection limit	Ref.
Based on molecular imprinted polymers	Tetracycline	Milk	CDs@MIPs	CDs@MIPs composites were directly coupled to Targets (amino-carboxyl condensation reaction between CDs and MIPs)	2.44 $\mu\text{g L}^{-1}$	[109]
	Enrofloxacin Danofloxacin	Milk	MIP (synthetic antibody mimic)	Direct distinguish in situ analytes bound to the MIP from the free analyte in solution via the FPI.	100.63 $\mu\text{g L}^{-1}$ 28.59 $\mu\text{g L}^{-1}$	[110]
Based on metal coordination	Oxytetracycline hydrochloride	Milk	CDs	The fluorescence quenching of CDs is caused by $\text{Fe}^{3+}$ ; fluorescence recovery after adding targets.	12.12 $\mu\text{g L}^{-1}$	[112]
	Chlortetracycline	Milk	Au/PtNCs	Fluorescence quenching response of Au/PtNCs toward chlortetracycline. And the recovery upon $\text{Al}^{3+}$ addition.	167.61 $\mu\text{g L}^{-1}$	[113]

also likely to compete with its aptamer for the adsorption sites on the AuNPs, meaning that pre-adsorbed kanamycin might inhibit the adsorption of its aptamer [103,104]. Thus, it is necessary to conduct control experiments with random DNA that cannot bind target molecules to ensure the proposed sensing mechanism.

Moreover, an interesting aptasensor was developed by the autofluorescence of the target, enrofloxacin (Fig. 6d). In the absence of the aptamer, the fluorescence of enrofloxacin was quenched by adsorption on graphene oxide (GO). In the presence of the aptamer, the enrofloxacin-aptamer complex had a lower affinity for GO and the fluorescence intensity increased. This aptasensor was developed to detect enrofloxacin in milk with a calculated LOD of 3.7 nM [94].

### 5.3. Sensors based on split aptamers

An interesting feature of aptamers is its programmability. For example, an aptamer can be split into two pieces and binding can still occur when both pieces are present. However, this type of splitting is more difficult for antibodies. Split aptamers provide additional methods for sensing. As shown in Fig. 7, binding of targets can shorten the distance of the two split aptamer fragments, resulting in a fluorescence signal change. Using the detection of kanamycin as an example, its split aptamer fragments were respectively labelled with CuS nanoparticles and biotin which could couple with streptavidin coated paramagnetic particles. The presence of kanamycin allowed magnetic separation of the CuS nanoparticles, which were then dissolved by concentrated nitric acid. The generated Cu(II) ions were reduced to Cu(I) by sodium ascorbate to act as a catalyst for the click chemistry and produce a fluorophore. The detection range was 0.04–20 nM [97].

### 5.4. Sensors based on structure-switching strategies

Since aptamers are also a piece of DNA, they can bind not only their target molecules but also their complementary DNA. Structure-switching aptamer based sensors are developed based on the competition of these two binding events [105]. The structure-switching strategy can be combined with FRET to avoid the centrifugation step. Both GO [98] or AuNPs [100] are used as quenchers (Fig. 8a1). The fluorescent assays for oxytetracycline and kanamycin detection in milk were presented with LODs of 0.01 mM [98] and 321 pM [100], respectively. Furthermore, researchers employed the single-

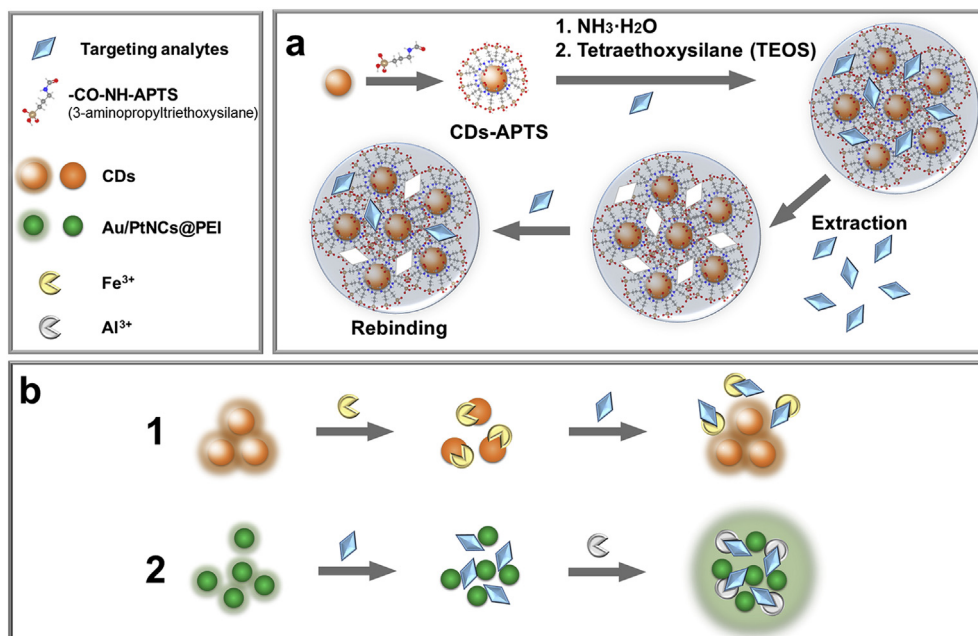


Fig. 9. (a) Schematic illustration of target recognition based on MIPs for the detection of veterinary drugs (figure created based on ref. [109]). (b) Schematic illustration of target recognition based on metal coordination for the detection of veterinary drugs (figure created based on ref. [112,113]).

stranded DNA-binding protein (SSB) as an alternative to the complementary chain. SSB was covalently linked to the fluorescent graphene oxide quantum dots (GOQDs) by amino-carboxyl condensation reaction (Fig. 8a2). A BHQ<sub>1</sub> (a dark quencher)-labeled aptamer was added to bind to the GOQDs via the SSB resulting in quenched fluorescence. Upon addition of the target, kanamycin, the quencher-labeled aptamer would dissociate from the GOQDs resulting in increased fluorescence. This method showed a LOD of 6 pg mL<sup>-1</sup> kanamycin in milk [52].

On top of that, a biosensor using the luminescence resonance energy transfer (LRET) from UCNPs to SYBR Green I dye was established [99]. As shown in Fig. 8b, the aptamers were hybridized with their complementary strands and covalently linked to UCNPs. SYBR Green I was inserted into the formed dsDNA duplex, and the fluorescence of the UCNPs could transfer to it. In the presence of the target oxytetracycline, SYBR Green I was released and the UCNP luminescence increased. Under optimal conditions, the LOD for oxytetracycline detection in milk was 0.054 ng mL<sup>-1</sup>.

Another type of the label-free structure-switching strategy is shown in Fig. 8c, where an aptamer was hybridized to amine-modified MSNs to block the pores, which were filled with rhodamine B [36]. In the presence of the target kanamycin, a significant

increase in fluorescence intensity was observed due to the separation of aptamers from complementary strands and the leakage of Rhodamine B. The saturated change of fluorescence signal was however only 12%. Both kanamycin and streptomycin showed a similar fluorescence increase, although this aptamer should only bind the former [106]. Therefore, nonspecific interactions could not be ruled out.

## 6. Target recognition based on molecularly imprinted polymers

In addition to aptamers and antibodies, molecular imprinted polymers (MIPs) represented another general method for molecular recognition [107]. MIP referred to the polymerization of selected functional monomers around a target molecule to imprint the shape of the target. After washing away the target, the resulting polymer cavity can selectively rebinding the target [108]. Production of highly stable MIP is cost-efficient and can be made in bulk quantity, although the selectivity is in general not as good as that of antibodies and aptamers. Some fluorescent biosensors using MIPs and other nanomaterials to detect veterinary drugs in food samples are shown in Table 4.

As shown in Fig. 9a, a CDs@MIP composite was synthesized by grafting MIP onto the surface of 3-



aminopropyltriethoxysilane (APTS)-modified CDs by amino-carboxyl condensation reaction. The CD@MIPs showed a high extraction efficiency for the targets tetracycline [109]. The concentration of tetracycline was quantitatively analyzed by fluorescence quenching (the fluorescent intensity of CDs@MIPs was quenched with increasing concentration of tetracycline) with a LOD of 5.48 nM in milk [109].

Another biosensor was developed for enrofloxacin and danofloxacin detection with the LODs of 0.28 and 0.08  $\mu\text{M}$  in milk, respectively [110]. As fluoroquinolones, these two drugs exhibited inherent fluorescence, which was widely used for their quantitation [111]. The free drug molecules had a low polarization value, while the polarization increased upon binding to their MIPs, allowing their detection.

## 7. Target recognition based on metal coordination

Many drug molecules have metal binding groups and such metal coordination interactions have also been harnessed to develop fluorescent sensors when combined with nanomaterials. In some systems, both metal ions and target molecules can affect the fluorescence of nanomaterials. As shown in Fig. 9b1, the catechol groups on the surface of water-soluble fluorescent CDs can bind  $\text{Fe}^{3+}$  ions and render fluorescence quenching. The quenched fluorescence was recovered with the addition of oxytetracycline due to the competition coordination [112]. This “turn-on” sensing strategy was expected to enhance the sensitivity because of a low background signal, and it was used for the detection of oxytetracycline hydrochloride with a LOD of 24.4 nM in milk. However, it is likely that other ligands with a strong affinity for  $\text{Fe}^{3+}$  can also produce a similar signal increase.

In another example, a highly method was developed by using Au/Pt nanoclusters (NCs) capped with polyethyleneimine (Au/PtNCs@PEI) acting as a fluorescent nanoprobe. As shown in Fig. 9b2, the fluorescence was quenched upon addition of the target chlortetracycline, which was believed to aggregate the nanoprobe. The fluorescence of Au/PtNCs@PEI gradually weakened as the concentration of the target increased. A biosensor was developed for chlortetracycline detection in milk with a LOD of 0.35  $\mu\text{M}$  [113]. Similar quenching was also observed in the presence of tetracycline, oxytetracycline and doxycycline. Interestingly, when  $\text{Al}^{3+}$  was added,

only the chlortetracycline sample showed a strong fluorescence recovery.

## 8. Conclusions and future prospective

In this review, we summarized various molecular recognition mechanisms coupled with nanomaterials for designing fluorescent biosensors to detect veterinary drug residues in foods. Many of these sensors were reported to meet the requirements of high sensitivity and accuracy, and sometimes high throughput detection and easy operation, which are critical for rapid, convenient, and quantitative detection. The detection strategies are very versatile, and can employ various target recognition elements (antibodies, aptamers, MIPs), assisting reagents (coated antigens, complementary DNA chains, SSB), signaling elements (different kinds of fluorescent nanomaterials or quenchers), and signal conversion methods (FRET, LRET, IFE, ET). Despite these progresses, this field still faces many challenges based on the contents reviewed. For example, nanomaterials are inherently large and heavy, leading to slow diffusion and thus a long detection time. In addition, nonspecific adsorption by nanomaterials could adversely affect detection. Many of the reviewed work required sophisticated surface functionalization of nanomaterials. Combined with variation in their synthesis, reproducibility could be an issue. High quality yet easy to use fluorescent nanomaterials are desirable for this purpose.

The fluorescence immunoassays so far relied on competitive approaches. The antibody-based “sandwich method” is not suitable for small-molecule detection due to steric hindrances. However, the strategy based on the small size splitting aptamers seems to be able to solve this problem. Currently, high-quality aptamers for veterinary drugs are still quite limited and most examples were demonstrated using the kanamycin aptamer. We expect more work to be done on the selection of aptamers for the drugs. In addition, we recommend rigorous testing of aptamer-based sensors using non-binding random DNA sequences to ensure the designed sensing mechanisms and avoiding potential artifacts [103].

So far, most of the detection required sample pretreatment and extraction of drug molecules into a solution phase. By introducing new nanomaterials (such as near-infrared and upconversion nanomaterials), it might be possible to achieve directly in situ detection, which can significantly improve food analysis. Taking advantage of the sharp emission

peaks of many nanomaterials, in situ multiplexed detection would be even more desirable. Although some proof-of-concept work was performed with these materials, more rigorous and quantitative work on using these advanced optical materials and test the effect of various food matrix is needed.

The low matrix tolerance is a main bottleneck of the reported sensors leading to false-positive test results. For sample pretreatment, although immuno-affinity column are magnetic separation are commonly used to enrich the targets in the samples, these methods are quite expensive. MIPs appear to be a useful material for detection in food sample matrix, and this front can be further improved by combining MIP with aptamers [114]. With the rapid development of materials science and biotechnology, the development of novel, efficient and practical fluorescent biosensor systems based on nanomaterials are likely to be realized in the near future.

### Conflicts of interest

None declared.

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