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Advances in Aptamer-based Biosensors for Food Safety

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1. Introduction

The presence of unsafe levels of chemical compounds, toxins, and pathogens in food constitutes a growing public health problem that necessitates new technology for the detection of these contaminants along the food continuum from production to consumption. While traditional techniques that are highly selective and sensitive exist, there is still a need for simpler, more rapid and cost-effective approaches to food safety testing. Within this context, the field of food safety biosensors has emerged. Biosensors consist of a specific molecular recognition probe targeting an analyte of interest and a means of converting that recognition event into a measurable signal. As molecular recognition is the foundation of biosensing, there has been increased focus on the development of new molecular recognition probes for food-safety related molecular targets. Antibodies have been the gold standard in molecular recognition for several decades and have been incorporated widely into biosensors and assays relating to food (Ricci et al., 2007). Despite their applicability to food monitoring, they are not without their disadvantages, which are primarily linked to the requirement that antibody generation is an *in vivo* process. For example, highly toxic substances are not conducive to antibody generation. Furthermore, the batch to batch reproducibility of antibody generation can be less than satisfactory. Compounding these limitations is the fact that antibodies have short shelf-lives and can be challenging to chemically modify for incorporation into a biosensor platform. Nevertheless, the affinity and specificity of antibodies for their molecular targets make them convenient receptors for biosensing strategies.

Many of the disadvantages described above could be avoided with a molecular recognition probe of synthetic origin that would still maintain the required specificity and affinity. Because of their *in vitro* selection and production, the relatively new technology of aptamers has emerged as a viable alternative for use in biosensor platforms (Mascini, 2009). This chapter will focus on the recent literature in aptamers for food safety related targets, as well as the biosensor platforms in which these probes have been incorporated.

2. Aptamers in biosensing

Aptamers are single-stranded oligonucleotides that fold into distinct three-dimensional conformations capable of binding strongly and selectively to a target molecule. As molecular recognition probes, aptamers have binding affinities and specificities that are

comparable to, and in some cases even surpass those of monoclonal antibodies. For example, aptamers have been selected that have dissociation constants (Kd) in the nanomolar or picomolar range. Aptamers are discovered using an *in vitro* process called Systematic Evolution of Ligands by EXponential enrichment (SELEX), a procedure where target-binding oligonucleotides are selected from a random pool of sequences through iterative cycles of affinity separation and amplification (see Fig. 1) (Ellington & Szostak, 1990; Tuerk & Gold, 1990). The SELEX process begins with a large, random oligonucleotide library whose complexity and diversity can be tailored through its distribution and number of random nucleotide regions (Luo et al., 2010). These sequences are exposed to the molecule of interest and those with an affinity for the target are separated from non-binding sequences. The elution of the binding sequences from the target and the polymerase chain reaction (PCR) amplification of those binders yields an enriched pool for subsequent, more stringent, selection rounds. After several rounds, this pool is cloned, sequenced, and characterized to find aptamers with the desired properties.

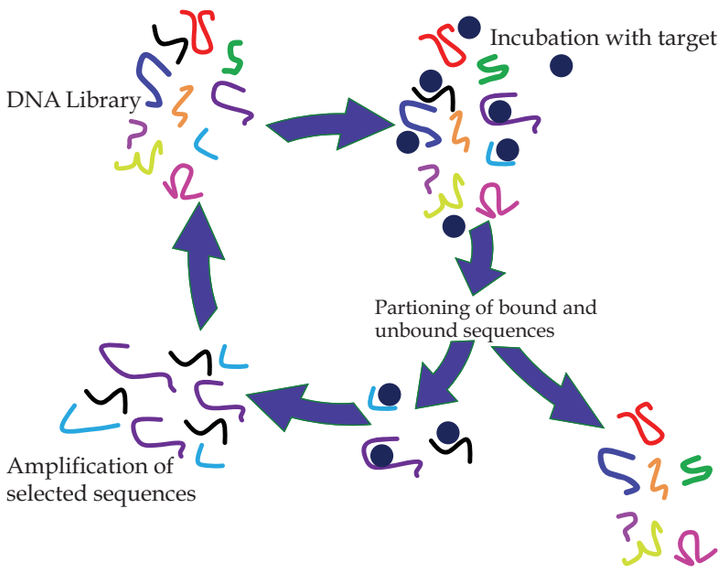


Fig. 1. Schematic overview of the SELEX procedure for the selection of aptamer sequences.

Targets for which aptamers can be developed are varied and range from small molecules (Huizenga & Szostak, 1995), to proteins and even whole cells. The *in vitro* nature of the selection process allows for the discovery of aptamers for even non-immunogenic or highly toxic substances. In addition to this advantage, aptamer technology offers several other benefits over antibodies. First, high-purity aptamers can be chemically synthesized at a low cost and can be easily modified with dyes, labels, and surface attachment groups without affecting their affinities. Second, aptamers are more chemically stable under most environmental conditions, have a longer shelf life, and can be reversibly denatured without loss of specificity. These properties make aptamers attractive in the development of low-cost, robust diagnostics and biosensors.

An examination of the development of aptamers and aptamer-based biosensors for food safety related targets can be found below. The chapter will be divided into two main parts: aptamers for small molecule food contaminants and aptamers for food safety-related pathogens.

Target Class	Target	DNA/ RNA	K_d (nM)	Biosensor Platform	LOD	Ref
Antibiotics	Chloram-phenicol	RNA	2100	-	-	(Burke et al., 1997)
Tetracyclines	Tetracycline	RNA	1000	-	-	(Berens et al., 2001)
		DNA	64	Electrochemical	10 nM	(Niazi, Lee Gu, 2008)
		DNA	-	Electrochemical	1 ng/mL	(Zhang et al., 2010)
	Oxytetra-cycline	DNA	10	-	-	(Niazi, Lee, Kim et al., 2008)
				Electrochemical	-	(Y. S. Kim et al., 2009)
				Colorimetric	-	(Y. S. Kim et al., 2010)
Amino-glycosides	Streptomycin	RNA	1000	-	-	(Wallace & Schroeder, 1998)
	Tobramycin	RNA	30-100	-	-	(Goertz, Colin Cox Ellington, 2004a)
		RNA	20000	-	-	(Morse, 2007)
		RNA	9	-	-	(Y. Wang & Rando, 1995)
		2′-OMe-RNA		Electrochemical (Impedence)	0.7 µM	(González-Fernández et al., 2011)
	Kanamycin	RNA	180	-	-	(Kwon et al., 2001)
		RNA	10-30	-	-	(Goertz, Colin Cox Ellington, 2004b)
	Neomycin	RNA	Low nM	-	-	(Goertz, Colin Cox Ellington, 2004b)
		RNA	1800	-	-	(Cowan et al., 2000)
				Electrochemical (Impedence)	"sub µM"	(de-los-Santos-Alvarez et al., 2007)
		2′-OMe-RNA		Surface Plasmon Resonance (SPR)	10 nM	(de-los-Santos-Álvarez et al., 2009)
Mycotoxins	Ochratoxin A	DNA	200	-		(Cruz-Aguado & Penner, 2008a)
				Fluorescence Polarization	5 nM	(Cruz-Aguado & Penner, 2008b)
				Electrochemical	30 pg/mL	(Kuang et al., 2010)
				Electrochem-iluminescent	0.007 ng/mL	(Z. Wang et al., 2010)
				Colorimetric	20 nM	(Yang et al., 2010)

Target Class	Target	DNA/ RNA	K_d (nM)	Biosensor Platform	LOD	Ref
				Strip (Fluorescence)	1.9 ng/mL	(L. Wang et al., 2011a)
				Strip (Chromato-graphic/ Absorbance)	0.18 ng/mL	(L. Wang et al., 2011b)
				Electrochemical	0.07 ng/mL	(Bonel et al.,)
				Fluorescence (quenching)	22 nM	(Sheng et al.,)
	Fumonisin B1	DNA	100			(McKeague et al., 2010)
Food packaging	Bisphenol A	DNA	-	Electrochemical (Sandwich Carbon Nano-tube sensor)	10 fM	(Lee et al., 2011)
		DNA	-	-	-	(Okada et al., 2003)
Adulterants	Sulfor-hodamine	DNA	190	-	-	(Wilson & Szostak, 1998)
		RNA	70	-	-	(Holeman et al., 1998)
	Melamine	DNA		Resonance Scattering	10-15 ng/L	(Z. Jiang et al., 2011)
Pesticides *Herbicides*	Atrazine	DNA	2000	-	-	(Sinha et al., 2010)
Fungicides	Malachite Green	RNA	<1000	-	-	(Grate & Wilson, 1999)
			600	Fluorescence	-	(Stead et al., 2010)
Inorganic ions	Arsenic (3^+, 5^+)	DNA	5 (3^+), 7 (5^+)			(M. Kim et al., 2009)
	Mercury (2^+)	DNA	-	Colorimetric	0.6 nM	(Li et al., 2009)
				Resonance Scattering	34 fg/mL	(Z. Jiang et al., 2010)
				Fluorescence	220 μM	(Xu et al.,2010)

Table 1. Table of aptamers and aptamer-based biosensor platforms for small-molecule targets.

2.1 Aptamers for small molecule food safety targets

Targets that can be classified under this category include high priorities for food analysis such as pesticides, toxins, veterinary drugs and other contaminants that may be present in a wide array of food products. Table 1 lists aptamers and aptamer-based biosensor platforms that have been developed for these targets. Section 2.1 will briefly highlight these systems and three promising biosensor platforms that target small molecules will be discussed in Section 2.2.

2.1.1 Antibiotics

In addition to their use in the treatment of bacterial diseases in humans and animals, antibiotics are important in animal husbandry because they significantly enhance growth when added to animal feed. However, the accumulation of antibiotics in food-producing animals is a potential cause of the increasing occurrence of antibiotic resistance. For this reason, European Union (EU) legislation has forbidden the practice of adding antibiotics to animal feed since 2006. As a result, fast, sensitive methodologies are being developed and used by food-safety control laboratories to ensure the control of antibiotic residues in live animals and animal products (Cháfer-Pericás et al., 2010).

While several antibiotic families are used in veterinary medicine and are tested in foods, only a handful of aptamers have been developed that recognize them. As seen in Table 1, RNA aptamers have been developed against chloramphenicol and several aminoglycosides such as streptomycin, kanamycin, tobramycin and neomycin. In particular, several aptamers have been developed for members of the tetracycline family. An RNA and DNA aptamer exist that recognize tetracycline as well as a DNA aptamer for oxytetracyline. Several of these have been integrated into electrochemical based testing systems. Overall, these antibiotic aptamers have a wide range of affinities for their targets, having dissociation constants from micromolar to low nanomolar. However, the detection systems that have been developed that use these aptamers all display detection limits in the nanomolar range.

2.1.2 Mycotoxins

Toxic fungal metabolites known as mycotoxins can contaminate a wide range of agricultural commodities and are high priority targets for the development of new molecular recognition probes and biosensors. It is estimated that at least 25% of the grain produced worldwide is contaminated with mycotoxins. Problematically, even small concentrations of mycotoxins can induce significant health problems including vomiting, kidney disease, liver disease, cancer and death (Cheli et al., 2008). The first mycotoxin aptamer was developed for Ochratoxin A (OTA). This toxin is produced by *Aspergillus ochraceus* and *Penicillium verrucosum* and is one of the most abundant food-contaminating mycotoxins in the world (De Girolamo et al., 2011). OTA is a nephrotoxic toxin, with strong carcinogenic effects on rodents, as well as documented teratogenic and immunotoxic effects in humans (Cruz-Aguado & Penner, 2008a). Since its development in 2008, this aptamer has been integrated into several biosensor detection systems including electrochemical, electrochemiluminescent, colorimetric and fluorescent platforms as well as chromatographic and fluorescent test strips. More recently, an aptamer for fumonisin B_1 (FB_1) has recently been developed. Fumonisin mycotoxins are produced by *Fusarium verticillioides* and *F. proliferatum* species, fungi that are ubiquitous in corn (maize). Insect damage and some other environmental conditions result in the accumulation of fumonisins in corn-based products worldwide. FB_1 is a nephrotoxin in all species tested, a carcinogen in rodents and a reproductive toxicant in rodents and likely in humans (Bolger et al., 2001). Both the FB_1 and the OTA DNA aptamers bind to their target with nanomolar dissociation constants.

2.1.3 Heavy metals

Inorganic metals contaminate foodstuffs including fish and fish products, meat and meat products, milk and milk products, eggs, fats and oils as well as animal feeds. Arsenic is a toxic carcinogen found in many parts of the world. It can exist in four oxidation states (-3, 0,+3, and+5); however, arsenate [As(V)] and arsenite [As(III)] are the most abundant.

Human exposure can occur through direct ingestion, such as drinking arsenic contaminated water, and through indirect ingestion such as consuming crops grown from arsenic-accumulated soils. Both acute and chronic health effects may result, the more serious effects include cancer, skin lesions, arsenicosis and cardiovascular diseases (M. Kim et al., 2009). Kim *et al*. developed a high affinity DNA aptamer for arsenic that can bind to arsenate [(As(V)] and arsenite [As(III)] with a dissociation constant of 5 and 7 nM respectively (M. Kim et al., 2009).

Mercury ion (Hg^{2+}) is highly toxic and a widespread contaminant. It is a potent neurotoxin that damages the central nervous and endocrine system. In addition, fish and shellfish concentrate mercury in their bodies, often in the form of methylmercury. The presence of mercury in fish can be a health issue, particularly for women who are or may become pregnant, nursing mothers, and young children (Xu et al., 2010). Routine detection of mercury is central for evaluating the safety of aquatically derived food supplies. As shown in Table 1, several label-free DNA aptamer based sensors have been developed for the direct detection of Hg^{2+}.

2.1.4 Food packaging

Contaminants migrating from food packaging have been detected in several food related matrices including foodstuffs, food stimulants, and food contact materials and articles. Bisphenol A (BPA), known to be an endrocrine disruptor since the 1930s, is used as a monomer compound in polycarbonate plastic products. Major concerns regarding the use of bisphenol A in consumer products were reported by the media in 2008. In 2010, a report from the United States Food and Drug Administration (FDA) raised further concerns regarding BPA exposure to fetuses, infants and young children. In September 2010, Canada became the first country to declare BPA as a toxic substance and to ban its use in baby bottles (US FDA, 2010). As seen in Table 1, several aptamers and aptamer platforms have been developed in response to these concerns. For example, Lee *et al.* developed an aptamer-sandwich based carbon nanotube sensor able to detect BPA at very low concentrations (Lee et al., 2011).

2.1.5 Adulterants

After the first report in 2003 indicating the illegal presence of the dye Sudan I in some foods in the European Union (EU), there have been many reports of the presence of illegal dyes in chili powder, curry powder, processed products containing chili or curry powder, sumac, curcuma and palm oil. These dyes are often both genotoxic and carcinogenic (EFSA, 2005). One such illegal dye is rhodamine B. While there is no known aptamer that recognizes rhodamine B, there is both a DNA and RNA aptamer that recognizes and binds to sulforhodamine B (Holeman et al., 1998; Wilson & Szostak, 1998).

Melamine is another chemical adulterant that is sometimes illegally added to food samples to increase the apparent protein content. Codex guidelines set the maximum amount of melamine allowed in powdered infant formula to 1 mg/kg and the amount allowed in other foods to 2.5 mg/kg. An aptamer-based resonance scattering assay has been developed for melamine detection. The oligo-T sequences with melamine binding affinity, however, were not determined through SELEX, but rather designed based on the idea that multiple hydrogen bonding interactions could support formation of an aptamer-melamine complex. While the design of this aptamer raises some concern about potential specificity problems, the authors confirmed that common metal ions, amino acids and proteins do not interfere with the assay (Z. Jiang et al., 2011).

2.1.6 Pesticides

Malachite Green (MG) has been used in aquaculture since 1936. It is a fungicide approved for use in aquarium fish in Canada; however, it is not approved for use in fish intended for human consumption. MG has been used for the treatment of external fungal and parasitic infections on fish eggs, fish and shellfish. It is also a very popular treatment against the common fresh water fish disease, ichthyophthirius (Stead et al., 2010). However, it has been determined that eating fish contaminated with malachite green poses a significant health risk. Scientific evidence indicates that a metabolite of malachite green (LMG), leucomalachite green, may be a genotoxic carcinogen that persists in fish tissues. A malachite green aptamer was developed in 1999 (Grate & Wilson, 1999). This aptamer has been recently used by Stead *et al.* as the recognition element in a fluorescence-based screening assay for MG detection in fish tissue (Stead et al., 2010). (See Section 2.2.1)

Atrazine is one of the most heavily used herbicides in Canada and the United States, as it is used as a pre- and post-emergence weed control agent for corn and rapeseed. Atrazine is a persistent environmental pollutant and widespread contamination of groundwater has been reported in the United States. It is associated with birth defects, menstrual problems and cancer when consumed by humans (Health Canada, 1993). Although it has been excluded from a re-registration process in the European Union, it is still one of the most widely used herbicides in the world. Sinha *et al.* used a combination of *in vitro* and *in vivo* selection to develop a synthetic riboswitch (an aptamer sequence coupled to an expression platform that can regulate a gene upon binding) to atrazine (Sinha et al., 2010).

2.2 Small molecule food safety targets case studies

As seen in Table 1, there are several biosensor platforms that are used for the detection of small molecular food contaminants. Here, we highlight a few recent sensors from the literature that have been used and tested directly in food samples.

2.2.1 Fluorescence-based detection of malachite green in fish tissue

Malachite green (MG) is a triphenylmethane dye used as a fungicide, antiparasitic and antiprotozoan agent in the treatment of farmed fish. Leucomalachite green (LMG) is the primary metabolite and is predominant to MG *in vivo* and highly persistent in edible fish tissue. Despite the potential health risk MG and LMG pose to humans, surveillance programs have identified their continuing incidence in farmed fish samples. For example, there was over 100 notifications under the EU Rapid Alert System for Food and Feed (RASFF) regarding the illegal use of MG between 2003 and 2007 (Stead et al., 2010). Current screening methods must be able to detect total MG and LMG concentrations at or below 2μg/kg. Unfortunately, typical detection methods for MG are time consuming and include high-performance liquid chromatography HPLC with visible detection or liquid chromatography–mass spectrometry LC-MS. Prior to the novel aptamer-based procedure, there has been limited progress in the development of rapid screening methods. This screening procedure, developed by Stead et al., is the first reported use of an RNA aptamer based detection system for the detection of chemical residues in food.

In 1999, Grate et al. developed a 38-mer RNA aptamer (see Fig. 2) that bound to MG with a dissociation constant (K_d) of less than 1μM (Grate & Wilson, 1999). The K_d value for the aptamer produced for use in this study was estimated to be approximately 600 nM, assuming a 1:1 binding model.

While MG has a low fluorescence quantum yield due to its facile vibrational de-excitation, a greater than 2000 times fluorescence enhancement was noted when bound by the aptamer.

Electrostatic forces and base stacking upon binding with the aptamer causes the aromatic rings of MG to adopt a more coplanar, rotationally stabilized structure, leading to this effect.

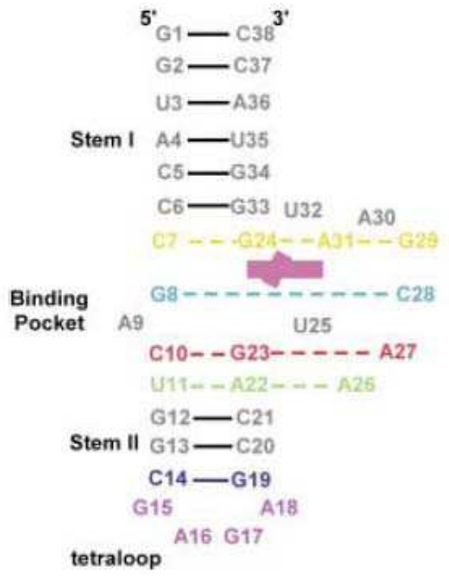


Fig. 2. Secondary stucture of the malachite green-RNA complex with the MG binding pocket indicated. Yellow indicates a base quadruple, red and green mark base triples, cyan the G8-C28 base pair and purple a GNRA tetraloop. The base pair adjacent to the tetraloop is shown in dark blue, the other stem base pairs are colored grey (Flinders et al., 2004). Reproduced with permission from Wiley-VCH.

The fluorescence signal associated with the RNA-MG complex was determined by excitation at a wavelength of 618nm and measuring emission at 643nm. The binding interaction produced a clear linear relationship with fluorescence intensity over the range of 0-40nM of MG. The pH operational range was determined to be between pH 5 and 9 for the RNA-MG complex, where the optimal fluorescent signals being observed between pH 6 and 7.5. A concentration of 5-10mM of Mg2+ and the presence of some monovalent cation (either Na+ or K+) was found to be required for the stability of the complex. Formation of this complex was time and temperature dependent. Maximum binding was achieved after 16 minutes at ambient temperature and remained stable for an hour. At temperatures outside this range, the formation of the ligand binding pocket was likely disrupted and signals were not fully recoverable. However, the complex at 0°C, once formed, was the most stable, surviving for about 4 weeks.

In order to perform the MG assay, fish tissues were extracted using acetonitrile. Since total LMG and MG is required for food safety detection, the ability of the RNA aptamer to detect LMG was investigated. However, addition of LMG to the RNA aptamer resulted in no change in fluorescence intensity. Due to this apparent lack of binding, an oxidation procedure was incorporated following extraction to ensure LMG was oxidized to the MG form. A final clean-up was then performed using solid-phase extraction (SPE) cartridges prior to analysis. Testing was performed on several spiked fish tissue samples including *Salmo trutta* and *Oncorhynchus mykiss* spp. and *Salmo salar*. Strong fluorescent signals were obtained in the positive samples compared to the blank controls. The threshold concentration of MG at which the signal was detectable was 5nmol/L (2ng/mL), a value

sufficient for RASFF testing standards. It was estimated that a batch of 20 samples can be prepared and analyzed within 4 hours and it is possible to automate the SPE steps. Therefore, this novel RNA aptamer testing system has proven to be a successful rapid detection and semiquantitative method for direct MG detection in fish tissue.

2.2.2 Aptamer-based strip assay for toxin detection

Ochratoxins are dangerous by-products of several fungal species, mainly in the *Aspergillus* and *Penicillium* genera, which can contaminate foods and beverages including cereals, beans, nuts, spices, dried fruits, coffee, cocoa, beer, and wine. Ochratoxin A (OTA) is nephrotoxic and carcinogenic and poses a serious threat to the health of both humans and animals. The International Agency for Research on Cancer has classified OTA as a possible carcinogen to humans. Based on risk assessment performed by the Joint Food and Agriculture Organization/WHO Expert Committee on Food Additives (JECFA), over 50% of human exposure to OTA is a result of exposure from cereals and cereal products. Regulatory limits for OTA exist in several countries, and testing of products is carried out at central testing laboratories. For example, the European Commission established a maximum limit of 5 µg/kg for raw cereal grains and 3 µg/kg for all cereal-derived products (De Girolamo et al., 2011). In 2008, Cruz-Aguado et al. developed the first mycotoxin aptamer for OTA. The selected DNA aptamer bound to OTA with a nanomolar dissociation constant and displayed high selectivity (Cruz-Aguado & Penner, 2008a).

With this sequence, Wang et al. developed a chromatographic strip assay method for rapid OTA detection (L. Wang et al., 2011b). The aptamer-based strip assay was based on the competition for the aptamer modified with gold nanoparticles (GNPs) as the visual reporter between ochratoxin A and DNA probes. GNPs were prepared and thiolated aptamers (Aptamer: 5'-GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA AAA AAA AAA AAA AAA AAA-SH-3') were self-assembled on the nanoparticle surface. DNA probes (Test line DNA probe 1: 5'-Biotin-CTA GCC CAC ACC CAC CGC ATT TCC CTC GTA GCC TGT-3' Control line DNA probe 2: 5'-Biotin-TTT TTT TTT TTT TTT TTT-3.) were conjugated to streptavidin using the 5′ biotin. The strip was assembled as follows (see Fig. 3). The nitrocellulose membrane, glass-fiber membrane (conjugated pad), sample pad and absorbent pad were layered in sequence and pasted onto a plastic backing plate. The streptavidin–DNA probe 1 and streptavidin–DNA probe 2 conjugates were deposited onto the strip to form the test line and control line. Finally the GNP–aptamer probe was added to the glass fiber membrane.

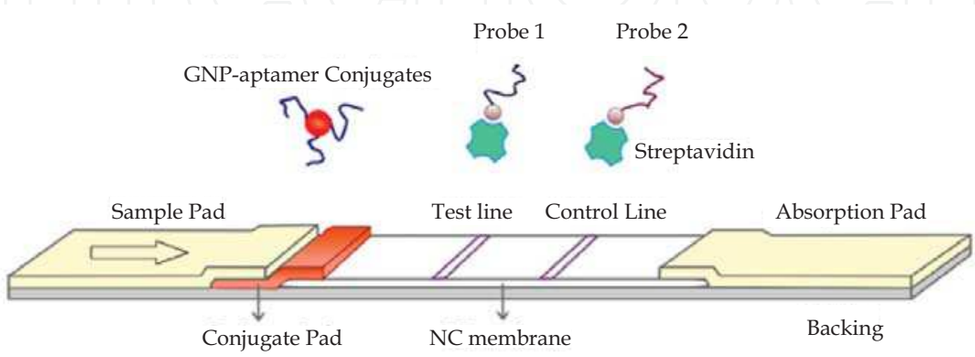


Fig. 3. Construction of the aptamer-based strip. Reproduced with permission from Elsevier.

This strip relied on the competitive reaction between the DNA probe 1 (test line) and the target OTA. In the presence of OTA, the aptamer-GNP could not hybridize to the DNA probe 1 in the test line, thus causing the red color intensity to become weaker. Regardless of the concentration of OTA, the aptamer–GNP probe could hybridize with DNA probe 2 in the control line to ensure the validity of the detection test (see Fig. 4).

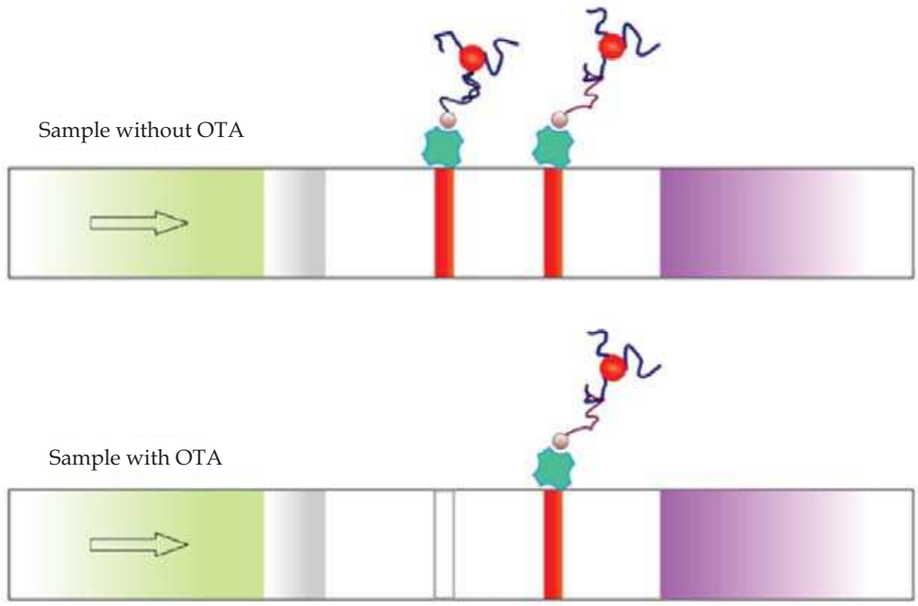


Fig. 4. Schematic of the detection principle of the strip. Reproduced with permission from Elsevier.

When using this strip for qualitative purposes, it was found to have a visual limit of detection (LOD) of 1ng/mL. However, it is possible to use this strip for semi-quantitative purposes. Using a scanning reader, a quantitative calibration curve was constructed. Based on this, the quantitative LOD was 0.18ng/mL which is better than the antibody-based strip method and comparable to the ELISA methods used for detection. Specificity of this strip assay was also tested using the mycotoxins deoxynivalenol, fumonisin B_1, zearalenone and microcystin-LR and was found to be specific for OTA. These fabricated strips were stable after 30 days. In addition, OTA analysis was performed on spiked wine samples. The recoveries were from 96% to 110% which met the detection requirements. Overall, this semi-quantitative assay proved to be rapid (less than 10 minutes), inexpensive and reliable in the detection of OTA.

2.2.3 Tetracyline determination in milk using an aptamer-modified electrode

Tetracycline is a naturally occurring, broad-spectrum polyketide antibiotic produced by species of *Streptomyces*. Tetracycline has been a popular and economically valuable drug for the last six decades as it can be easily isolated by fermentation. It is used against many bacterial infections due to its ability to inhibit protein synthesis. For these reasons, tetracyline has been widely used in human therapy, aquaculture and veterinary medicine. It is also extensively used as an animal growth promoter (Berens et al., 2001). As a result, tetracycline has been detected in food products, such as meat, milk, eggs and chicken.

Therefore, there is an increase in demand to discover novel methods for fast tetracycline detection that are applicable to food matrices. Zhang et al. developed one such method using a DNA aptamer modified glassy carbon electrode (Zhang et al., 2010). While there are several aptamers that have been developed for tetracycline (as seen in table 1), the authors performed a new selection to develop DNA aptamers for this assay. These modified glassy carbon electrodes were amino-functionalized using 3-aminopropyltriethoxysilane chemistry, allowing the aptamers to be tethered to the surface using 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride/ N-hydroxysulfosuccinimide (EDC/NHS) chemistry. The cyclic voltammetry (CV) measurements were performed using the aptamer-modified electrode with a standard three electrode system in the presence of $K_3Fe(CN)_6$ as a redox reporter. In the absence of tetracycline, a $Fe_3(CN)_6^{3-}/Fe_2(CN)_6^{4-}$ redox couple was measured due to its interaction with the electrode surface. In the presence of tetracyclines, the aptamer binds to the target, blocking the electrode surface and interrupting the redox system. This leads to a decrease in peak current. Peak current vs. concentration was measured and plotted as a standard curve for samples in phosphate buffered saline as well as directly in milk (see Fig. 5). A linear relationship between the tetracycline concentration and the current was found between 0.1–100ng/mL, and a LOD of 1 ng/mL was achieved. There was no significant response from penicillin, demonstrating the specificity of the system. Finally, six concentrations of tetracyclines in milk samples were analyzed using the electrode biosensor. The measured concentration vs. the actual concentration showed less than a 10% coefficient of variation, indicating the biosensor produces precise results. This biosensor proved to be a reliable, sensitive, inexpensive and specific detection system for tetracycline in milk samples. In addition, detection time was only 5 minutes; therefore, this system could prove useful in food safety testing.

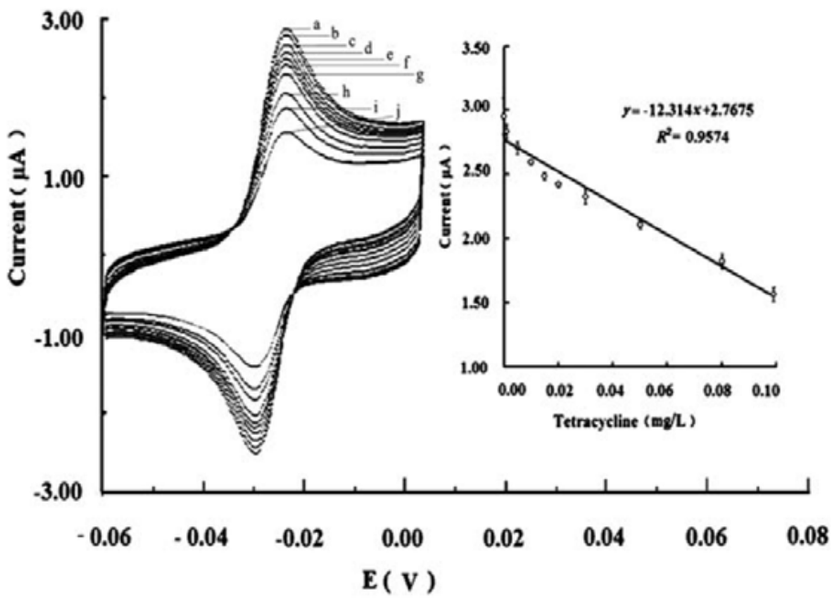


Fig. 5. Cyclic voltammograms of the tetracycline milk determination. The tetracycline concentrations are (a) 0mg/L (b) 0.001 mg/L (c) 0.005 mg/L (d) 0.01 mg/L (e) 0.015 mg/L (f) 0.02 mg/L (g) 0.03 mg/L (h) 0.05 mg/L (i) 0.08 mg/L (j) 0.1 mg/L. The inset is the plot of the tetracycline concentration versus the peak current at 0.02V. Reproduced with permission from RSC.

2.3 Aptamers for pathogenic food safety targets

Foodborne pathogens are implicated in millions of illnesses and thousands of deaths on a yearly basis. In addition to the clear health risk associated with contaminated foods, there is also the often devastating economic impact to the food producer that must be considered. As a result, rapid, accurate and sensitive assays and biosensors for bacteria, viruses, and other similar targets are of significant interest. Table 2 lists aptamers and aptamer-based biosensor platforms that have been developed for these targets.

Target Class	Target	DNA/ RNA	K_d	Biosensor Platform	LOD	Ref
Bacteria	*Bacillus Thuingiensis* (spores)	DNA	-	Fluorescence (Quantum dots)	1000 cfu/mL	(Ikanovic et al., 2007) (Bruno & Kiel, 2002)
	Campylobacter Jejuni	DNA	-	Fluorescence (Magnetic Bead/Quantum dot sandwich)	10-250 cfu in food matrix; 2.5 cfu in buffer	(Bruno et al., 2009)
			-	Capillary Electrophoresis Immunoassay	6.4×10^6 cells/mL	(Stratis-Cullum et al., 2009)
	Escherichia Coli DH5α strain	RNA	-	Single-walled Carbon Nanotube (SWNT) Field Effect Transistor (FET)	-	(So et al., 2008)
	CECT 675 cells	RNA	-	Electrochemical (SWNT Potentiometry)	6 cfu/mL (milk) 26 cfu/mL (apple juice)	(Zelada-Guillen et al., 2010)
	Crook's strain (8739)	DNA	-	Fluorescence Resonance Energy Transfer (FRET) and Surface Plasmon Resonance (SPR)	30 *E. Coli* units/mL	(Bruno et al., 2010)
	ETEC K88 fimbriae protein	DNA	25-44 nM	Fluorescence Assay	-	(Li et. al., 2011)
	Francisella tularensis - japonica (bacterial antigen)	DNA	25 ng (K_a)	Aptamer-Linked Immobilized Sorbent Assay (ALISA)	1.7×10^3 bacteria/ mL	(Vivekananda & Kiel, 2006)
	Listeria monocytogenes (internalin A protein)	DNA	84 nM	Fibre-optic sensor (Aptamer and Antibody-modified)	1000 cfu/mL	(Ohk et al., 2010)
				Magnetic beads	1 cfu/mL	(Yamamoto et al., 2010)

Target Class	Target	DNA/ RNA	K_d	Biosensor Platform	LOD	Ref
	Salmonella Typhimurium (outer membrane proteins)	DNA	-	Magnetic beads	<10 cfu/g (spike and recovery)	(Joshi et al., 2009)
	(Type IVB pilus protein)	RNA	9 nM	-	-	(Pan et al., 2005)
	Staphylococcus aureus	DNA	480 ± 210 nM	Confocal microscopy of infected samples	-	(Cao et al., 2009)
	Yersenia (Yop51)	RNA	18 nM, 28 nM	-	-	(Bell et al., 1998)
Viruses	Apple-Stem pitting virus	DNA	PSA-H protein: 8 nM; MT32 protein: 55 nM	Surface Plasmon Resonance	250 nM	(Lautner et al., 2010)
				Double Oligonucleotide Sandwich Enzyme-Linked Oligonucleotide Assay (DOS-ELONA)	100 ng/mL	(Balogh et al., 2010)
Other	Botulinum neurotoxin	DNA	3-51 nM	-		(Tok & Fischer, 2008)
	Egg-white lysozyme (allergen trigger)	DNA	3 nM	-	-	(Tran et al., 2010)
				Electrochemical (voltammetry)	0.5 µg/mL	(Cheng et al., 2007) (Kirby et al., 2004)
		RNA	-	Fluorescence (microarray)	70 fM	(Collett et al., 2005)

Table 2. Table of aptamers and aptamer-based biosensor platforms for pathogen and macromolecular targets.

Section 2.3 will describe these systems briefly, while three biosensor platforms targeting pathogens in food will be discussed in Section 2.4.

2.3.1 Bacteria

Foodborne illnesses and outbreaks are commonly caused by bacteria. Visible symptoms of infection typically do not appear until about 12-72 hours after food consumption. Thus, it is important to be able to detect these pathogens rapidly, at very low levels in complex food matrices, as early as possible in the food continuum to minimize the risk of illness and to avoid product recalls. In response to these needs, a number of aptamers and aptamer-based sensor platforms have been developed and demonstrate much potential in food safety applications.

Some strains of the gram-positive bacteria *B. Cereus* are responsible for foodborne illnesses causing severe nausea, vomiting and diarrhea. While the presence of large numbers of *B. cereus* (greater than 10^6 organisms/g) in a food can lead to illness, the US FDA recommends that *B. cereus* in infant formula does not exceed 100 cfu/g. Ikanovic et *al.* selected a DNA aptamer for *Bacillus thuingiensis* (BT) spores with eight rounds of selection. The aptamer was then applied to produce a novel, solution-based sensor by using quantum-dot reporter molecules for detection via fluoresence spectroscopy. BT spores are closely related to the food poisoning agent *B. cereus* so detection of BT demonstrates potential cross-over applications to its foodborne relative.

C. jejuni, a species of gram-negative bacteria commonly found in animal feces, is one of the most common causes of human gastroenteritis and has been linked with subsequent development of Guillain-Barré syndrome. A dose as low as 400-500 organisms can cause infection. DNA aptamers for *Campylobacter jejuni* have been incorporated into sensing platforms (Bruno et al., 2009; Stratis-Cullum et al., 2009). The former used a sandwich assay, comprised of aptamers conjugated to both magnetic beads and red quantum dots, in order to detect the bacterial protein in food matrices (see section 2.4). The latter implements a capillary electrophoresis immunoassay to characterize the bacterial cells.

Detection of *E. Coli* O157:H7 is of high priority in food surveillance as infection with this gram-negative bacteria can lead to hemorrhagic diarrhea, and occasionally kidney failure, especially in vulnerable populations such as infants and the elderly. Sensitive methods are particularly important here as the infectious dose can be as low as 10-100 organisms. Several DNA and RNA aptamer-based platforms have been developed for strains of food-borne *E.coli.*, which serve as models for O157:H7 detection. One example is an aptamer-modified single-walled carbon nanotube in both field-effect transistors (So et al., 2008) and label-free potentiometry measurements (see section 2.4) (Zelada-Guillen et al., 2010) to provide rapid, reusable biosensors. Additionally, a FRET-based assay using a DNA aptamer for *E. coli.* has been developed (Bruno et al., 2010).

Francisella tularensis–japonica is a bacterial pathogen that causes tularemia in humans and can be transmitted through contact with infected animals. With an estimated infectious dose of 1 bacillus, signs of the infection in the form of skin lesions occur within 3-5 days. Other signs include, fever, lethargy, anorexia and potentially death. A DNA aptamer cocktail for this bacteria was selected with 10 rounds of SELEX. Upon testing in a sandwich Aptamer-Linked Immobilized Sorbent Assay (ALISA) and dot blot analysis, the aptamer cocktail, which contained 25 unique sequences, exhibited good binding specificity in its ability to recognize only the bacteria in comparison to *Bartonella henselae*, pure chicken albumin or chicken lysozyme (Vivekananda & Kiel, 2006). Thus, it appears that this novel anti-tularemia aptamer cocktail may find application as a detection reagent for *F. tularensis* (Jones et al, 2005).

Listeria Monocytogenes is a virulent foodborne bacteria that causes hundreds of deaths in the US annually. The FDA, USDA and EU have all implemented a zero-tolerance rule for *L. monocytogenes* in ready-to-eat foods, leading to a substantial effort to develop very sensitive biosensors for the pathogen. Aptamer-based sensors have been developed to detect components of the *Listeria monocytogenes* bacteria. One platform uses magnetic beads conjugated to DNA aptamers for target detection and also investigated therapeutic applications for this platform. Another platform currently developed involves an antibody-aptamer functionalized fibre optic sensor that detects *L. monocytogenes* cells from

contaminated ready-to-eat meat products such as sliced beef, chicken and turkey (Yamamoto et al., 2008, 2009, 2010).

Salmonella enterica is among the most commonly encountered bacteria and a prominent cause of foodborne illness around the world. Most frequently found in contaminated beef and poultry, the infectious dose for the enteric subspecies *typhimurium* is approximately 10^4 bacilli (Srinivasan et al, 2004). It is a notable target of interest as it has been demonstrated to have a multi-drug resistance. Joshi and co-workers (2009) have reported a DNA aptamer-based platform for capturing and detecting *S. typhimurium* outer membrane proteins at low levels using magnetic beads. Conversely, an RNA aptamer was selected for the IVB Pili protein of S. *typhimurium,* which is linked to the pathogenesis of the bacteria. The 70-nt sequence was determined to have low nanomolar binding constant a stem-loop secondary structure. Further studies found that the aptamer could also significantly inhibit the protein into human monocytic cells (THP-1) (Pan et al., 2005), demonstrating its potential as a therapeutic.

The U.S. FDA estimates an infectious dose of 10^5-10^6 bacilli for *Staphylococcus aureus* (Schmid-Hempel & Frank, 2007). This bacteria is well known for causing a wide range of illnesses from minor wound infections to major diseases such as pneumonia. Staphylococcal food poisoning can cause symptoms such as nausea, vomiting, retching and abdominal cramping. Whole-bacteria subtractive SELEX was performed on *Staphylococcus aureus* to yield an aptamer cocktail of five sequences (Cao et al., 2009). As determined by competition experiments and flow cytometry, the sequences were effective in binding to various strains of the bacteria in a mixture.

Yersinia enterocolitica, a rare foodborne bacteria, has a relatively higher infectious dose than the above mentioned bacteria (~10^9 bacteria) but nevertheless produces similar clinical symptoms including diarrhea, mild fever and abdominal pain. It is often found in undercooked pork but also in unpasteurized milk and untreated water. One promising study comes from Bell *et al.* (1998) where two aptamers were selected for the PTPase enzyme, Yop51, which has been considered a virulent determinant for *Yersinia*. The sequences were characterized with nanomolar binding and found to effectively inhibit the enzyme, which have important implications in viral replication. It was found that the two sequences shared a 21-residue motif and bound specifically to Yop51 over a homologous PTPase called rat PTP1.

2.3.2 Viruses

Despite the existence of pathogenic foodborne viruses, few virus-targeting aptamers have been developed. Aptamers for food viruses such as Apple Stem-Pitting Virus (ASPV) have also demonstrated their significance in the food industry. Although these viruses may not necessarily be contracted by humans, they compromise the integrity and appearance of crops in agricultural development, which ultimately impacts their marketing value and can result in a loss of revenue. This aptasensor example (Lautner et al., 2010) and its applicability will be discussed in section 2.4.

2.3.3 Other macromolecular targets

There are other targets that do not fall under the classification of bacteria or viruses that are relevant for food safety detection. One example is the botulism neurotoxin. This protein is associated with *Clostridium botulinium,* a common contaminant can in canned foods that have been improperly prepared (e.g. heated before canning). The neurotoxin has a very

lethal dose (~ng of bacteria) and is considered one of the most poisonous substances known. Tok & Fischer (2008) selected a DNA aptamer for two botulinum neurotoxin-related targets, BoNT-toxoid and BoNT Hc-peptide. Using single microbead SELEX, the study resulted in high-affinity sequences that competitively bound to the target over corresponding antibodies. Another noteworthy example that has been studied over the past few years is egg-white lysozyme, a protein considered to be an allergen-trigger in consumers with egg allergies. A few promising aptasensor platforms developed so far include label-free voltammetric assays (Cheng et al, 2007), Faradaic impendance spectroscopy (Rodriguez et al., 2005) and RNA microarray technology (Collett et al., 2005).

2.4 Pathogen case studies

As seen in Table 2, there are several biosensor platforms that may be applicable to real-world food testing. Here, we highlight a few recent aptamer-based sensors from the literature that have been used and tested directly in food samples.

2.4.1 Real-time potentiometric detection of bacteria in complex samples

One example of a feasible aptasensor for use in pathogen detection in food is the work presented by Zelada-Guillén and co-workers. They report the development of an electrochemical aptasensor using single-walled carbon nanotubes (SWNT) to selectively detect a non-pathogenic strain of *E. coli* cells to serve as a model for pathogenic strain O157:H7. Their motive for this aptasensor was to provide a simple, rapid and label-free means of detecting the target with comparably high sensitivity and selectivity.

The aptamer-based sensor was prepared by depositing SWNT on a polished glassy carbon surface. Amino-modifed aptamers for *E. coli* CECT 675 were tethered to the SWNT carboxyl groups via EDC/NHS chemistry. For *E. coli* CECT 675 testing in phosphate buffered saline (PBS), electromagnetic field (EMF) response was recorded to provide insight into the aptasensor's performance in terms of its stability and response. A rapid increase in EMF signal was observed from 4 cfu/mL to ~10^4 cfu/mL with 50% of the response achieved within seconds. The signal remained stable over almost 2 hours. Upon regeneration of the sensor, the EMF baseline remained the same and it was determined that regeneration could be done up to five times before the detection limit and sensitivity were compromised. The selectivity tests showed no cross-reactivity indicating that the aptamer not only had preference for its target but it could also discriminate the particular *E.coli* strain. In order to detect the bacteria in real beverage samples, the ionic strength of the sample needed to be controlled through a prefiltration step to prevent a false-positive response. The biosensor was tested on apple juice and skim milk samples that had been spiked with with *E. coli* CECT 675 and were compared to control samples of the liquid matrices (Fig. 6). Specificity was confirmed through controls of buffer, juice and milk contaminated with *E. coli* CECT 4558, *Lactobacillus casei* CECT 4180 and *Salmonella enterica* CECT 409. The aptasensor was exposed to increasing concentrations of bacteria. EMF measurements were made with a change in concentration every 10 seconds. Simultaneous measurements using the plate count method and the MacConkey agar test were conducted for experimental validation. The limit of detection for CECT 675 in milk was determined to be 6 cfu/mL and in apple juice was 26 cfu/mL. These detection limits are comparable with European regulated limits demonstrating the sensor's applicability in food testing. Incorporating O157 aptamers in a similar biosensor format therefore holds a great deal of potential.

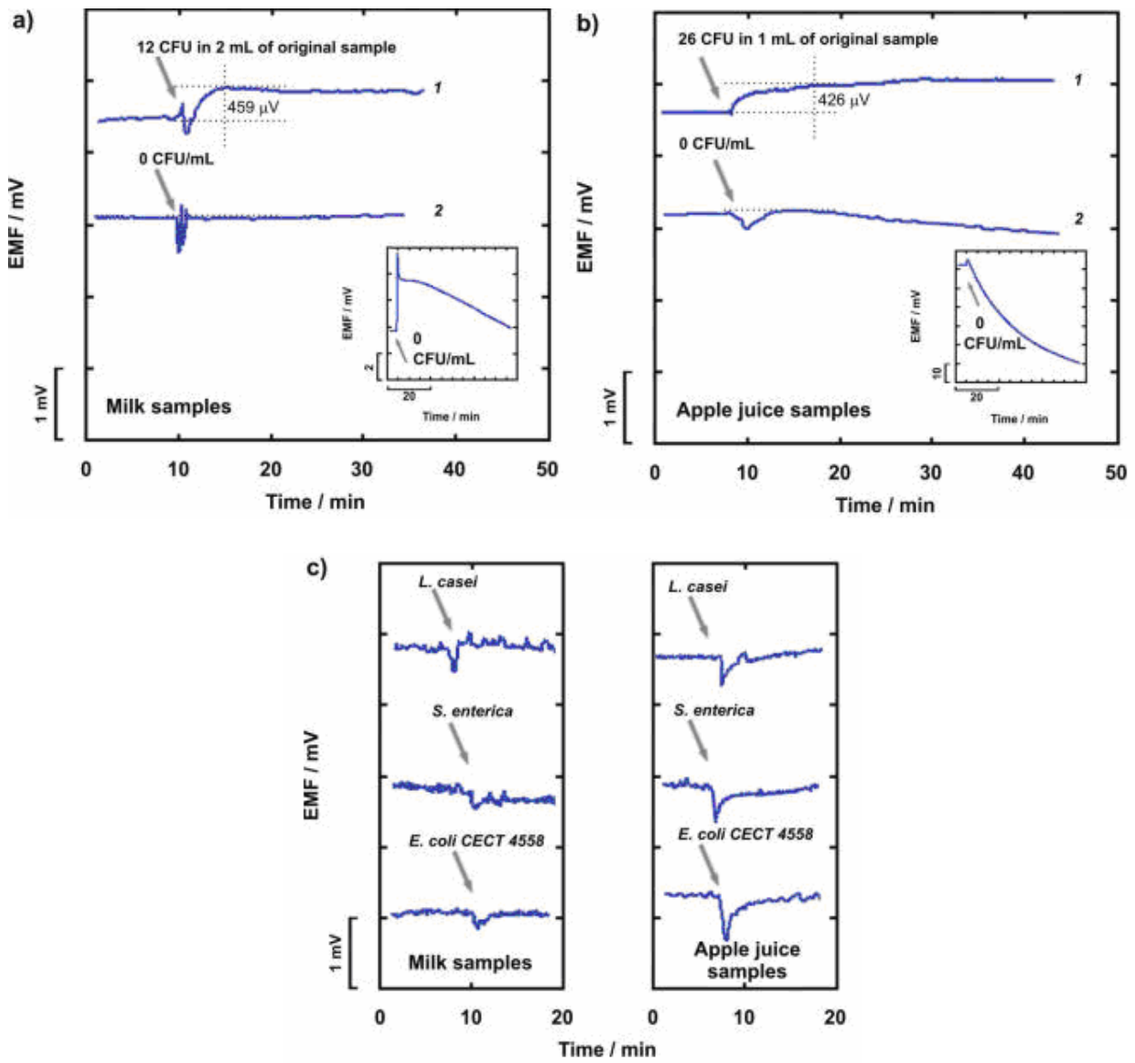
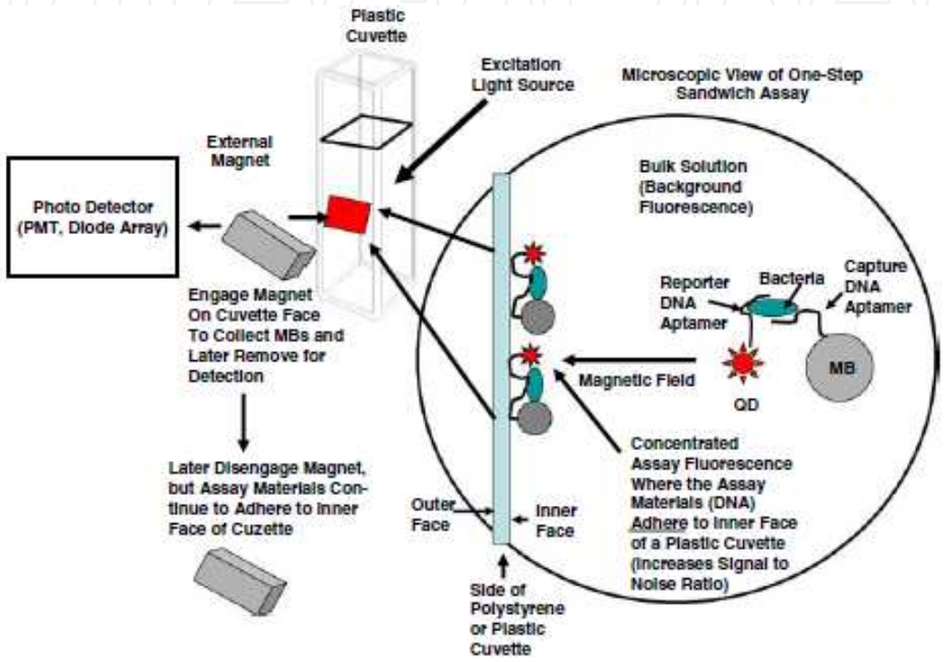


Fig. 6. Detection of microorganisms in liquid matrices via potentiometry. (a) Sample of skim milk containing 12 cfu of *E. coli* CECT 675 (1) compared to uninfected milk (2). (b) Sample of apple juice containing 26 CFU of *E. coli* CECT 675 (1) compared to uninfected juice. Insets in both graphs demonstrate the importance of pretreatment of these samples to reduce false positive results caused by other charged species in the samples. (c) Selectivity studies in milk (left) and apple juice (right) samples with 10^3 CFU/mL of various bacteria (*L. casei, S. enterica, E. coli* CECT 4558). Reproduced with permission from ACS.

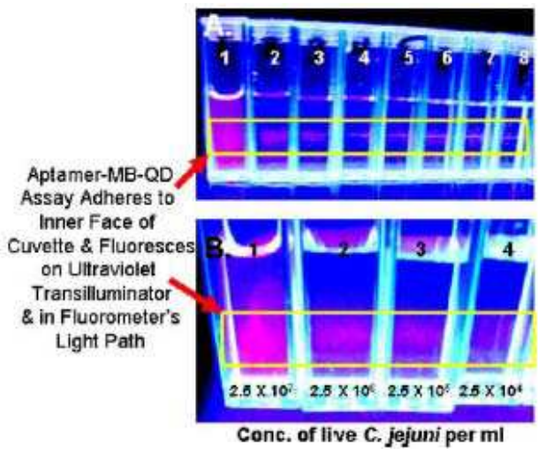
2.4.2 Plastic-adherent DNA aptamer-magnetic bead and quantum dot sandwich assay for *Campylobacter* detection

Another example of a promising aptasensor for food safety is presented by Bruno *et al.* where they have developed a sandwich assay for *Campylobacter jejuni*. This concept of a an assay built from aptamer functionalized magnetic beads and quantum dots is based on their previous assay developed for BT spores (found in Table 2). The schematic of the assay is illustrated in Fig. 7. Two amino functionalized aptamers, designated C2 and C3 were prepared. C2 was conjugated to magnetic beads to behave as the capture probe, while C3

was conjugated to red CdSe/ZnS quantum dots to behave as the reporter probe. Equivalent amounts of the two aptamer particles were added to a 1cm polystyrene cuvette. Live and heat-killed *C. jejuni* bacteria were transferred to the cuvettes and with the facilitation of a magnet, the probes were led towards the inner face of the cuvettes allowing them to adhere to the surface. This eliminated the need for washing steps and allowed fluoresence to be measured in one plane with low background signal (Fig. 7). Moreover, the assay components could remain on the wall of the cuvette for up to a few weeks in sterile buffer at ambient temperature.



(a)



(b)

Fig. 7. (a) Schematic illustrating concept of sandwich assay for Campylobacter jejuni detection. (b) Assay of 10-fold serial dilutions of C.jejuni in binding buffer. Reproduced with permission from Springer.

Fluoresence emission from the quantum dot conjugated aptamer was measured and the detection limit in binding buffer was found to be 2.5 cfu/mL for both live and heat-killed *C. jejuni* bacteria (Fig. 8). Bacterial detection was also attempted in diluted food matrices such as 2% milk, chicken juice and ground beef wash. The detection limit for both live and heat-killed *C. jejuni* bacteria was determined to be in the range of 10-250 cfu/mL. In this case, a portable fluorometer could be effectively used as the fluorescence detector allowing this biosensor to serve as a practical field-based detection kit.

This aptasensor was also previously found to have very low cross-reactivity with other bacteria such as *E. coli* O157:H7, *L.monocytogenes* and *S.typhimurium.* In the present study, the aptasensor was further tested against different species of *Campylobacter* such as *C. coli* and *C. lari* in addition to other bacterial genera. The resulting cross-reactivity was observed to be very low for species outside the *Campylobacter* family but was fairly high for those within the genus, indicating potential use for detecting these other two pathogens.

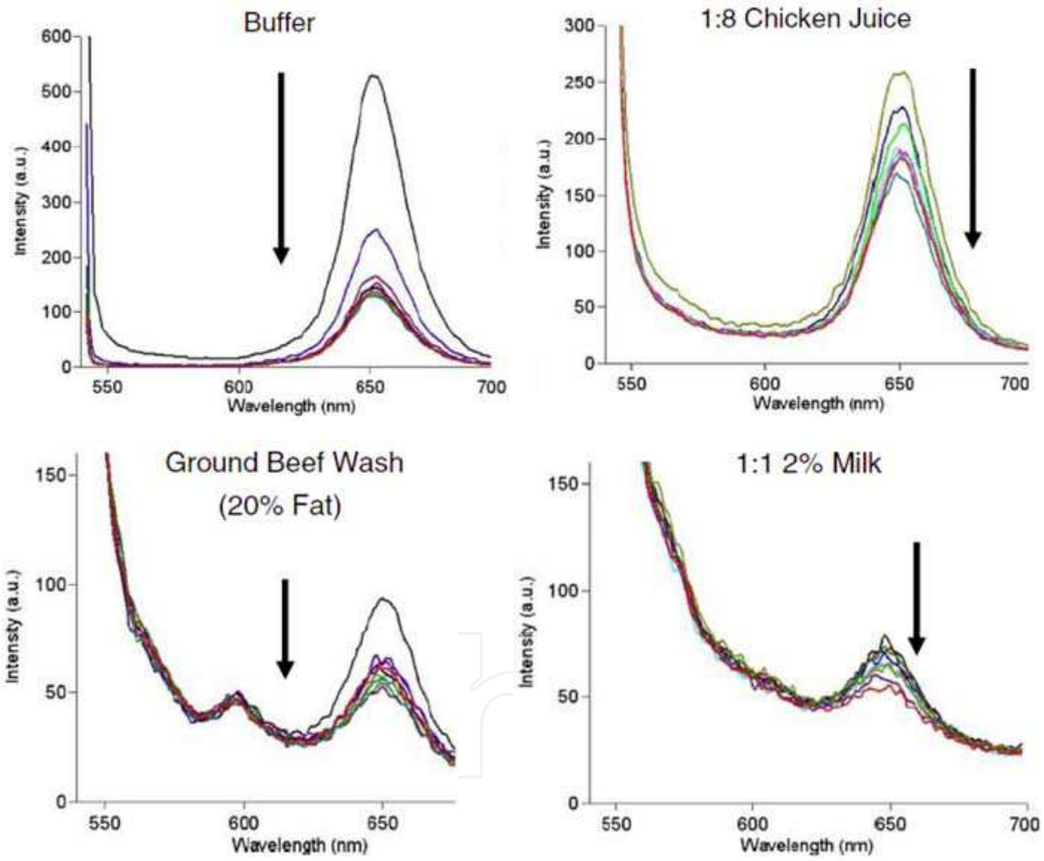


Fig. 8. Detection of C.jejuni bacteria in various food matrices using spectrofluorometry. Tenfold serial dilutions (using binding buffer as diluent) from 2.5 x 10^6 cfu/mL to 0 bacteria, indicated by the direction of the arrows, were measured. Reproduced with permission from Springer.

2.4.3 Aptamer-based biochips for label-free detection of plant virus coat proteins by SPR imaging

Apart from human health as a factor that is accounted for in food biosensors, food quality and integrity are also of importance. The appearance of food can influence the consumer's

decision to buy based on their perception of what "looks safe". Apple-stem pitting virus (ASPV) is a widely spread filamentous virus associated with growth disorders of pome fruits (e.g. apples and pears). Characteristics of the virus include yellowed veins and darkened spotted areas that appear charred. Current methods in detecting the virus such as real-time PCR are effective yet involve pre-treatment of samples and may become less sensitive when evolved strains of the virus are present. Thus, a label-free aptamer-based sensor for ASPV detection was proposed by Lautner and coworkers. Their sensor is composed of thiolated aptamers for ASPV coat proteins, PSA-H and MT32, tethered to a gold sensor chip surface (Fig. 9). Similarly, other sensor chips were prepared with random sequences for comparison. The sensor was tested with different concentrations of PSA-H and MT32 in PBS using Surface Plasmon Resonance (SPR) which allowed the determination of (K_d) for each aptamer. These were 55nM for the MT32 aptamer, 8nM for the PSA-H aptamer.

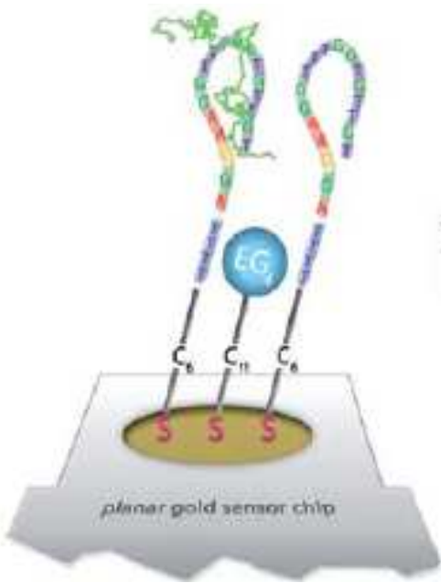


Fig. 9. Schematic of aptamer-based biochip for ASPV detection. Reproduced with permission from RSC.

Calibration curves were measured with the aptamer-functionalized surfaces and increasing concentrations of MT32 in apple leaf extract. The measurements on the graph were made relative to the surface prepared with a same-length random sequence. Specificity of the aptamer to its respective target was demonstrated and a nearly linear response was obtained. For detection of real ASPV-infected samples, ASPV-positive and ASPV-negative plant extracts were tested. Fig. 10 shows the response curves for the samples at different dilutions. The SPR signal predictably decreases upon a decreasing concentration of virus, indicating that this is a feasible method for virus detection in real samples.

3. Conclusion

Aptamers are proving to be effective molecular recognition probes for the analysis of high priority food contaminants. Aptamer-based biosensor platforms generally show sensitivities and specificities that could allow them to be competitive with existing detection methods.

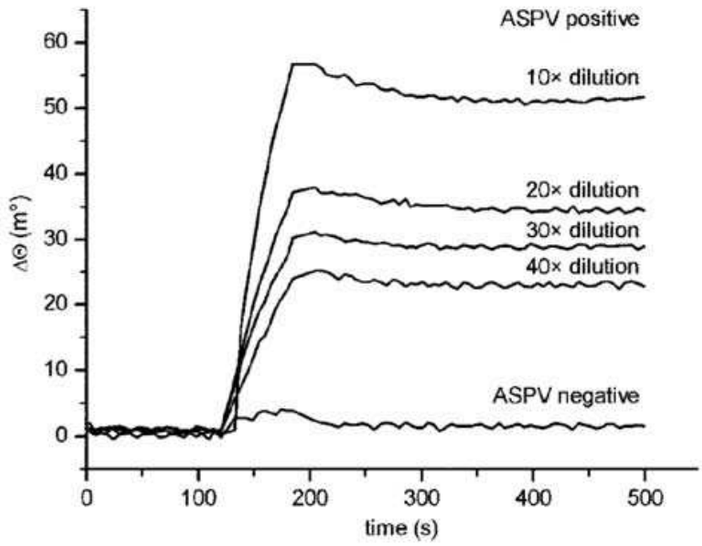


Fig. 10. SPR response curves of the MT32-based aptasensor detecting ASPV in apple leaf extracts at serial dilutions of total protein content. Reproduced with permission from RSC.

The *in vitro* nature of aptamer selection allows for a theoretically limitless variety of targets for which aptamers can be generated. This area of research is still in its infancy and the examples provided here represent only a small fraction of the potential aptamers which could be developed. In terms of small molecule targets, more focus should be paid to mycotoxins, given their prevalence and deleterious health effects. Similarly, aptamers for viral food pathogens are underrepresented, and should be investigated, particularly those related to gastroenteritis, such as rotaviruses and noroviruses. While several existing aptamer platforms have been tested in complex food matrices, it remains to be seen how many of these systems will perform under real-world conditions. Nevertheless, aptamer technology presents an opportunity for the development of robust, low cost, specific biosensors for food-safety applications.

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This book is a collection of contributions from leading specialists on the topic of biosensors for health, environment and biosecurity. It is divided into three sections with headings of current trends and developments; materials design and developments; and detection and monitoring. In the section on current trends and developments, topics such as biosensor applications for environmental and water monitoring, agro-industry applications, and trends in the detection of nerve agents and pesticides are discussed. The section on materials design and developments deals with topics on new materials for biosensor construction, polymer-based microsystems, silicon and silicon-related surfaces for biosensor applications, including hybrid film biosensor systems. Finally, in the detection and monitoring section, the specific topics covered deal with enzyme-based biosensors for phenol detection, ultra-sensitive fluorescence sensors, the determination of biochemical oxygen demand, and sensors for pharmaceutical and environmental analysis.

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