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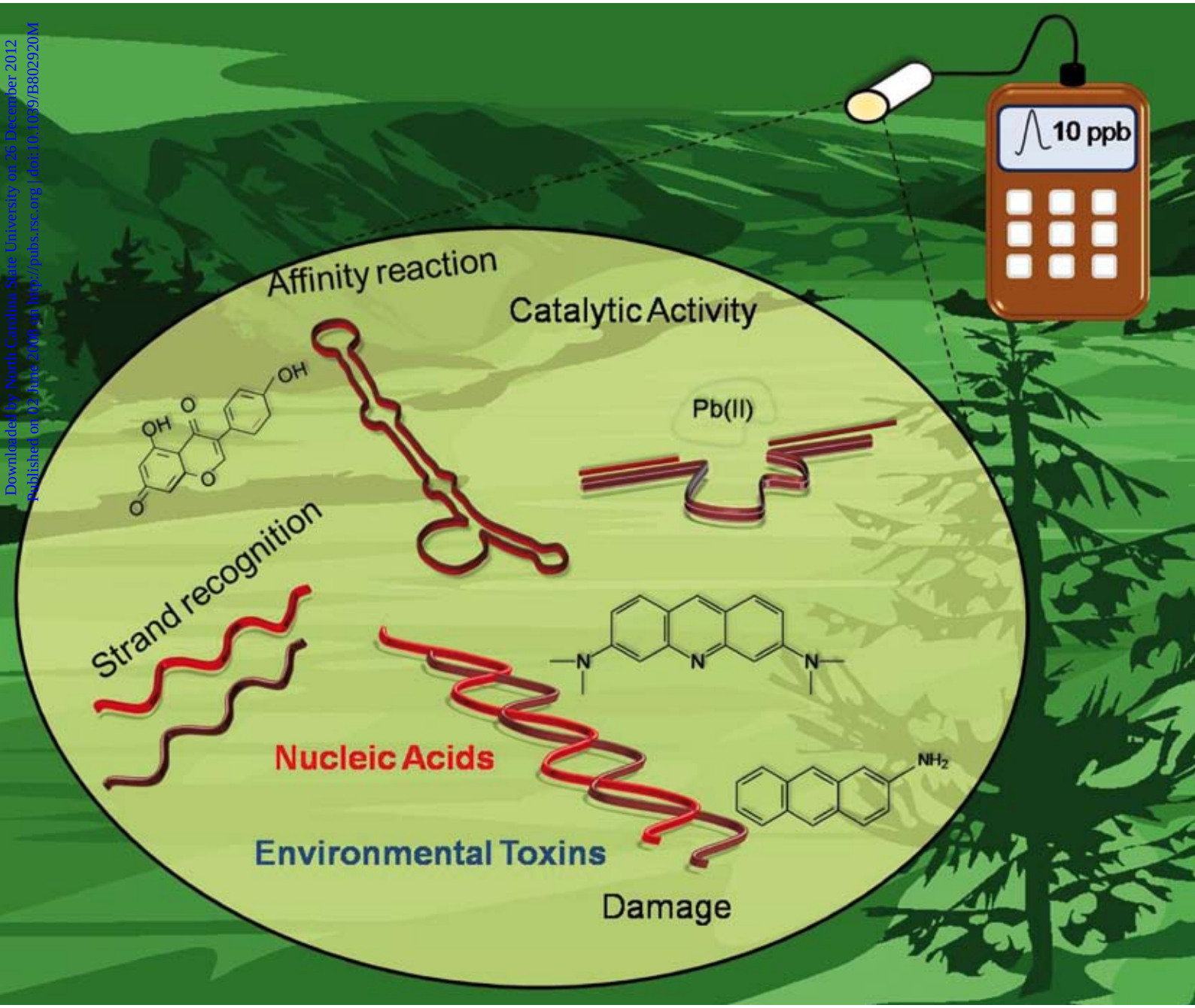

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Ilaria Palchetti and Marco Mascini*

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Nucleic acid-based biosensors are finding increasing use for the detection of environmental pollution and toxicity. A biosensor is defined as a compact analytical device incorporating a biological or biologically-derived sensing element either integrated within or intimately associated with a physicochemical transducer. A nucleic acid-based biosensor employs as the sensing element an oligonucleotide, with a known sequence of bases, or a complex structure of DNA or RNA. Nucleic acid biosensors can be used to detect DNA/RNA fragments or either biological or chemical species. In the first application, DNA/RNA is the analyte and it is detected through the hybridization reaction (this kind of biosensor is also called a genosensor). In the second application, DNA/RNA plays the role of the receptor of specific biological and/or chemical species, such as target proteins, pollutants or drugs. Recent advances in the development and applications of nucleic acid-based biosensors for environmental application are reviewed in this article with special emphasis on functional nucleic acid elements (aptamers, DNAzymes, aptazymes) and lab-on-a-chip technology.

1. Introduction: biosensors for environmental applications

Monitoring of contaminants in the air, water and soil is an instrumental component in understanding and managing risks to human health and ecosystems. The increasing number of potentially harmful pollutants in the environment calls for fast and cost-effective analytical techniques to be used in extensive monitoring programs. Given this requirement as well as the time and cost involved in the traditional chemical analysis of environmental samples (*e.g.* chromatographic methods), there is an expanding need for simple, rapid, cost-effective and field-portable screening methods.

Biosensors appear well suited to complement standard analytical methods for a number of environmental monitoring applications.[1,2]

A biosensor is defined by IUPAC[3] as a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with a transduction element. A biosensor should be clearly distinguished from a bioanalytical system, which requires additional processing steps. A device that is both disposable after one measurement, *i.e.* is single-use, and unable to monitor the analyte concentration continuously or after rapid and reproducible regeneration should be designated as a single-use biosensor.

The main advantages offered by biosensors over conventional analytical techniques are the possibility of miniaturization, portability and the ability to measure pollutants in complex matrices with minimal sample preparation. Although many of the systems developed cannot compete with conventional analytical methods in

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terms of accuracy and reproducibility, they can be used by regulatory authorities and by industry to provide enough information for routine testing and screening of samples.

Some excellent reviews have summarized the recent progress in the use of biosensors for environmental applications.[1,2,4–13] Different natural as well as synthetic biorecognition elements have been proposed in biosensing environmental pollutants. In recent years, nucleic acids (NAs) have been incorporated into a wide range of biosensors and bioanalytical assays, due to their wide range of physical, chemical and biological activities.[1,14,15]

As is commonly known, NA molecules have the function of carrying and passing genetic information. This can be exploited, from an analytical point of view for a specific identification of animal and vegetal species, genetically-modified organisms, bacteria, viruses, toxins, *etc*.

However, about 25 years ago, NA and in particular DNA (due to the fact that, compared with RNA, DNA molecules are less susceptible to hydrolysis and thus are highly stable) began to find a new role in the field of materials science and biotechnology.[16–19]

The intention of this article is to discuss recent advances and trends in the use of nucleic acid-based biosensors for environmental monitoring applications.

2. Nucleic acid-based biosensors

A nucleic acid (NA) is a macromolecule composed of nucleotide chains. The most common nucleic acids are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Nucleic acids are universal in living things as they are found in all cells and also in viruses. Traditionally, NA molecules were thought to have the sole function of carrying and passing genetic information from one generation to another.[16] Since the early 1990s, many NA molecules – known as aptamers – have been isolated from large libraries that are able to bind a broad range of molecules with high affinity and specificity.[20,21] The molecules that can be recognized by aptamers range from small organic molecules to proteins, cells and even intact viral particles.[19] A further advance in the development of function DNAs was made in 1994 when DNA was shown to act as a catalyst for the first time.[22] These catalytic DNA molecules are called DNAzymes (also described as DNA enzymes, deoxyribozymes or catalytic DNA). Moreover, DNAzymes and aptamers have been combined to form allosteric DNAzymes or aptazymes. These DNAzymes, aptamers and aptazymes are collectively called functional DNAs, whose functions extend beyond the Watson–Crick base pair recognition of complementary strands.[16]

Naturally-occurring NAs as well as DNA-templated materials that are responsive to chemical stimuli have been used to assemble biosensors.

An NA biosensor is defined as an analytical device incorporating an oligonucleotide, even a modified one, with a known sequence of bases, or a complex structure of NA (like DNA from calf thymus) either integrated within or intimately associated with a signal transducer. NA biosensors can be used to detect DNA/RNA fragments or either biological or chemical species. Most nucleic acid biosensors are based on the highly specific hybridization of complementary strands of DNA or RNA molecules; this kind of biosensor is also called a genosensor. In other applications (aptasensors), selected NAs play the role of a highly specific receptor of biological and/or chemical species, such as target proteins, pollutants or drugs. In addition, the interaction of chemical compounds with DNA molecules has been exploited for toxicity screening assays. Finally, catalytic RNA and DNA can be used to develop functional NA biosensors.

Various signal transduction mechanisms are used in NA biosensing applications: optical, electrochemical, mass, magnetic, and micromechanical.[23–25]

Optical detection by fluorescence spectroscopy is a popular method, due largely to the ease with which NAs can be fluorescently labeled, the availability of many different fluorophores and quenchers, and the inherent capability for real-time multiplex detection.[23] Chemiluminescence is another optical way to detect NAs. A different optical transduction, based on an evanescent wave device, can offer real-time label-free optical detection. These biosensors rely on monitoring changes in surface optical properties (a shift in resonance angle due to a change in the interfacial refractive index) resulting from the surface binding reaction.[23]

Electrochemical devices have also proven very useful, due to inherent miniaturization and their compatibility with advanced microfabrication technology.[24] Electrochemical detection usually involves monitoring a current response under controlled potential conditions. However, other changes in electrochemical properties such as capacitance, impedance and conductivity have been used.

Another useful label-free detection scheme relies on the use of quartz crystal microbalance (QCM) transducers. QCM biosensors consist of an oscillating crystal with a bioreceptor immobilized on its surface. The increased mass, associated with the biorecognition reaction, results in a decrease of the oscillating frequency. Acoustic wave sensors used in thickness-shear mode with a liquid sample detect changes in a number of physical properties including mass, viscosity and charge density.[23]

Micromechanical transduction based on cantilevers and label-free biosensors capable of detecting biomolecular interactions *via* the bending of microfabricated cantilevers coated with oligonucleotides were reported in the literature.[25]

In the present article, the literature regarding NA biosensors for environmental monitoring and toxic compound detection will be reviewed as NA-based affinity biosensors (genosensors and aptasensors), NA-based catalytic biosensors, and NA biosensors for the detection of chemically-induced DNA damage, thus considering the ligand more than the transducing principle.

3. NA-based affinity biosensors

Affinity biosensors allow the detection of affinity-based interactions between biomolecules, *e.g.* DNA hybridization or antibody–antigen (target–aptamer) recognition. The presence of target molecules in a sample can be verified by the binding of these molecules to their complementary bioreceptors, immobilized onto a transducer surface.

3.1 Genosensors

Genosensors result from the integration of a sequence-specific probe (usually a

short synthetic oligonucleotide) and a signal transducer. The probe, immobilized onto the transducer surface, acts as the biorecognition molecule and recognizes the target DNA or RNA.

The main steps to make a genosensor are probe selection and immobilization, development and detection of the hybridization reaction. An overview of those important aspects that contribute in creating successful genosensing devices are briefly described in the following section. However, for details we ask the reader to refer to specialist literature and several excellent reviews published on these topics in recent years,[26–38] since the aim of this review is the application of the genosensor for environmental monitoring. A dedicated session will address this issue.

3.1.1 Probe immobilization, hybridization and sample treatment. The specificity of the hybridization reaction is essentially dependent on the biorecognition properties of the capture oligonucleotide; thus, design of the capture probe is undoubtedly the most important pre-analytical step and a number of probes, variable for chemical composition and conformational arrangement, have been used to assemble genosensors. Most commonly, the probes are linear oligonucleotides [Fig. 1(A)], either synthesised *in situ* or pre-synthesised and afterwards immobilized onto the sensor surface. However, structured (hairpin) oligonucleotides are being used with increasing frequency.[38] Hairpin probes are structured oligonucleotides that combine a double-stranded stem region (due to intramolecular base pairing) and a single-stranded loop, which contains the capture sequence. Recently, probes produced by chemical changes to the backbone of naturally-occurring DNA or RNA are used more and more in NA sensing techniques. Among these, locked nucleic acid (LNA) and peptide nucleic acid (PNA) are the most used. LNA is a bicyclic nucleic acid where a ribonucleoside is linked between the 2′-oxygen and the 4′-carbon atoms with a methylene unit [Fig. 1(B)]. LNA can be used in any hybridization assay that requires high specificity and/or reproducibility. General properties of LNA oligonucleotides include highly stable base pairing with DNA and RNA,[39] exceptionally high thermal stability, improved discrimination, compatibility with most enzymes and predictable melting behaviour. Peptide nucleic acid (PNA) [Fig. 1(C)] is a synthetic nucleic acid reported in the early 1990s[40] that has an achiral neutral polyamide backbone formed by repetitive units of *N*-(2-aminoethyl)glycine linked to N bases. The PNA molecule that mimics DNA is advantageous as a probe molecule, owing to improved chemical and enzymatic stability relative to nucleic acids. The PNA molecule, being resistive to attack by nucleases, provides an extra edge over the use of conventional or naturally existing nucleic acids.

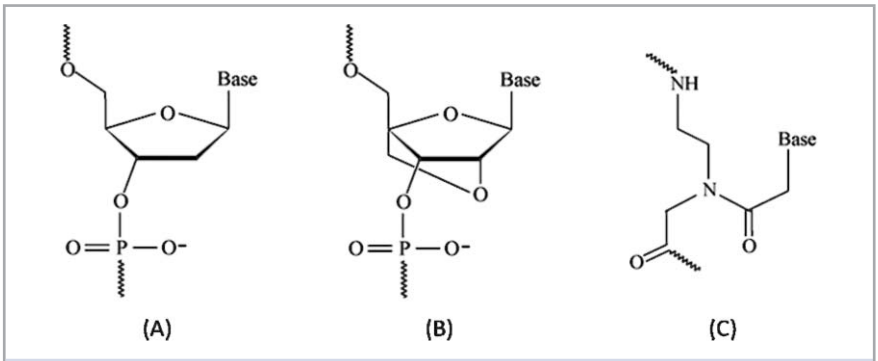

Fig. 1 DNA (A), LNA (B) and PNA (C) monomers.

The ability to immobilize the probe in a predictable manner while maintaining its inherent affinity for the target NA is crucial to the overall device performance. Hence, independently of the molecular identity and conformation of the probe, some general aspects must be taken into account when considering its surface attachment. Being obvious that the choice of the most appropriate immobilization protocol is strictly dependent on the characteristics of the transducing material (*i.e.* metal, glass, carbon surfaces), robust immobilization chemistries are usually preferred in order to prevent desorption of the probes from the sensing layer. Retention in a polymeric matrix, covalent attachment on a functionalized support, affinity immobilization [attachment of biotinylated probes to (strept)avidin-coated surfaces] and self-assembling (*i.e.* chemisorption of thiol-modified probes onto gold surfaces) are, to date, the most successful approaches.

Several variables affect the hybridization event at the transducer–solution interface and should be controlled carefully.[28] These include salt concentration, temperature, and the presence of accelerating agents, contact time and length of the probe sequence.[28] Then, once the target NA has been captured onto the sensor surface, a range of different approaches can be used for transducing the biorecognition event; these can be broadly divided into label-free and label-based schemes. Label-free approaches typically rely on the measurement of changes in the characteristics of the sensing layer, before and after the hybridization reaction. Piezoelectric as well as surface plasmon resonance (SPR) transduction are essentially label-free techniques. By contrast, when organic and organometallic electroactive compounds, nanoparticles, catalytic and redox enzymes are permanently bound [*e.g.* covalently or *via* (strept)avidin–biotin interactions] to one of the constituents of the surface-tethered duplex, the method will be considered as label-based. The sensitivity and reliability of label-based approaches are often still unrivalled.

Along with use of capture probes with improved selectivity and hybridization efficiency, specific pre-treatments of the samples can be used to greatly enhance the yield of the heterogeneous hybridization events and, consequently, the sensitivity of detection.

Detection thus depends on a pre-amplification of the target sequence. This is true for most genoassays and especially for DNA detection since RNA-based assays benefit from the typically high number of ribosomes per cell. The most commonly used pre-amplification techniques are the polymerase chain reaction (PCR) or nucleic acid sequence-based amplification (NASBA).

PCR is a technique widely used in molecular biology. It derives its name from one of its key components, a DNA

polymerase used to amplify (*i.e.* replicate) a piece of DNA by *in vitro* enzymatic replication. As PCR progresses, the DNA thus generated is itself used as a template for replication. This sets in motion a chain reaction in which the DNA template is exponentially amplified.

NASBA technology is based on the simultaneous enzymatic activity of reverse transcriptase, T7 RNA polymerase, and RNase in combination with two oligonucleotides. It depends on selective primer–template recognition to drive a cyclical, exponential amplification of the target sequence.[41]

3.1.2 Genosensors for environmental monitoring. Environmental applications of genosensors are in the field of species identification. For instance, genosensors were extensively exploited in the detection of pathogenic microorganisms relevant to food, biodefense and environmental contamination applications. Mainly, genosensors have been coupled to PCR, as a specific detection method of the amplified base sequence. Mo *et al.*[42] reported the detection of a few *Escherichia coli* cells per 100 mL of water, combining the detection of the *lac* gene with PCR amplification and quartz crystal microbalance (QCM) detection. QCM detection of PCR samples of *Aeromonas* strains, isolated from water, vegetables or human specimens were reported in ref. 43. The simultaneous electrochemical detection of *Salmonella enterica*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *E. coli* 0157:H7 amplicons in less than one hour was demonstrated in ref. 44, using a disposable electrode array. Wang[32] described electrochemical genosensors for *Cryptosporidium* as well as *E. coli*, *Giardia* and *Mycobacterium tuberculosis*. Lermo *et al.*[45] described a genomagnetic assay for the electrochemical detection of food pathogens based on *in situ* DNA amplification with magnetic primers. Electrochemical detection of harmful algae and other microbial contaminants in coastal waters using hand-held biosensors was demonstrated also in ref. 46. Baeumner *et al.*[47] detected as few as 40 *E. coli* cells per 1 mL sample using a simple optical dipstick-type biosensor coupled to NASBA, emphasizing the fact that only viable cells are detected and no false positive signals are obtained from dead cells present in a sample, which is important in respect to safety and also food and environmental sample sterilization assessments; similarly, a biosensor for the protozoan parasite *Cryptosporidium parvum* was developed.[48] Baeumner's group also developed a genosensor with integrated microfluidic system and a minipotentiostat for the quantification of Dengue virus RNA.[49] By combining microelectronics and microfluidics with the simple and effective liposome signal enhancement technology and an amperometric transducer, the authors designed a miniaturized electrochemical detection system (miniEC) that was easy to assemble and use. DNA and RNA molecules were quantified using a sandwich hybridization assay similar to the one previously described,[50,51] with the transduction mechanism being based on electrochemical rather than fluorescence detection.

A fast procedure that did not require any amplification of the targeted nucleic acids by PCR or NASBA was the one reported in Elsholz *et al.*,[52] where a low-density electrical 16S rRNA-specific oligonucleotide microarrays and an automated analysis system for the identification and quantization of pathogens, such as *E. coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *S. aureus*, and *S. epidermidis* were described.

Genosensors were also applied to the detection of genetically-modified plants or foods, such as the surface plasmon resonance (SPR) genosensors described by Feriotto *et al.* for the detection of Roundup Ready soybeans in which PCR-amplified sequences from transgenic and wild-type soybeans were investigated by an SPR sensor principle.[53] Examples of QCM or electrochemical biosensors are reported in refs 54 and 55.

However, in the literature many other examples of genosensor technology are reported. Although not specifically developed for environmental applications, the described technologies are pre-requisite for the development of DNA-based biosensors for environmental applications.

3.2 Aptasensors

Aptamers are single-stranded DNA or RNA ligands that can be selected for different targets starting from a huge library of molecules containing randomly created sequences.[19] The selection process is called systematic evolution of ligands by exponential enrichment (SELEX), first reported in 1990.[20,21] The SELEX process involves iterative cycles of selection and amplification starting from a large library of oligonucleotides with different sequences (generally 10^{15} different structures). After incubation with the specific target and the partitioning of the binding from the non-binding molecules, the oligonucleotides that are selected are amplified to create a new mixture enriched in those nucleic acid molecules having a higher affinity for the target. After several cycles of the selection process, the pool is enriched in the high-affinity sequences at the expense of the low-affinity binders.

The number of cycles required depends on the stringency of the conditions, but, once obtained and once the sequence is known, unlimited amounts of the aptamer can be easily achieved by chemical synthesis. In addition to this very important aspect of having an unlimited source of identical affinity recognition molecules available, aptamers can offer advantages over antibodies that make them very promising for analytical applications. The main advantage is the overcoming of the use of animals or cell lines for the production of the molecules. Antibodies against molecules that are not immunogenic are difficult to generate. By contrast, aptamers are isolated by *in vitro* methods that are independent of animals: an *in vitro* combinatorial library can be generated against any target. In addition, the generation of antibodies *in vivo* means that it is the animal immune system that selects the sites on the target protein to which the antibodies bind. The *in vivo* parameters restrict the identification of antibodies that can recognize targets only under physiological conditions, limiting the extension to which the antibodies can be functionalized and applied. Moreover, the aptamer selection process can be manipulated to obtain aptamers that bind a specific region of the target and with specific binding properties in different binding conditions. After selection, aptamers are produced by chemical synthesis and purified to a very high degree by eliminating the batch-to-batch variation found when using antibodies. By chemical synthesis, modifications in the aptamer can be introduced, enhancing the stability, affinity and specificity of the molecules. Often, the kinetic parameters of the aptamer–target complex can be

changed for higher affinity or specificity. Another advantage over antibodies can be seen in the higher temperature stability of aptamers and they can recover their native active conformation after denaturation; in fact, antibodies are large proteins sensitive to the temperature and they can undergo irreversible denaturation.

The large quantity of aptamers which have been selected to bind complex molecules of low molecular weight leads to the possible use of these aptamers not only in diagnostic assays, but also in a wider range of applications, such as environmental analytical chemistry. In spite of this, very few papers have been published reporting an application of aptamer-based biosensors for the detection of environmental analysis-related molecules. In particular, the potentialities of aptamers for this application have been reported after their selection,[56–58] but no direct application has been published so far. Selection of DNA ligands for the chloroaromatics 4-chloroaniline (4-CA), 2,4,6-trichloroaniline (TCA) and pentachlorophenol (PCP) was performed by a novel method utilising magnetic beads (MBs) having a linker arm for immobilization.[56] Moreover, Brockstedt *et al.*[57] reported for the first time the selection of RNA aptamers for the recognition of hydrophobic aromatic carcinogens. In particular, RNA aptamers with a K_d in the low micromolar range have been selected for aromatic amine residues using as a model methylendianiline, which is a widely-used industrial chemical employed to manufacture plastics, glues and foams. Gu and Famulok[58] described the selection of specific RNA aptamers against microcystin-LR: four clone RNAs were identified with relatively high affinity to microcystin-LR (with microcystin-LR concentration as low as 0.5 mmol L^{-1}). These new RNA aptamers were identified by the authors as a new way for future application in the environmental monitoring of microcystin.

Among those aptamers that have actually been applied to the detection of target molecules for environmental analysis, we can find a 17β-estradiol DNA aptamer (Fig. 2).[59] This 76-mer DNA aptamer, with a K_d of 0.13 mM for 17β-estradiol, has been selected and used for the development of an electrochemical biosensor for the detection of 17β-estradiol,[59] a well-known endocrine disrupting molecule.

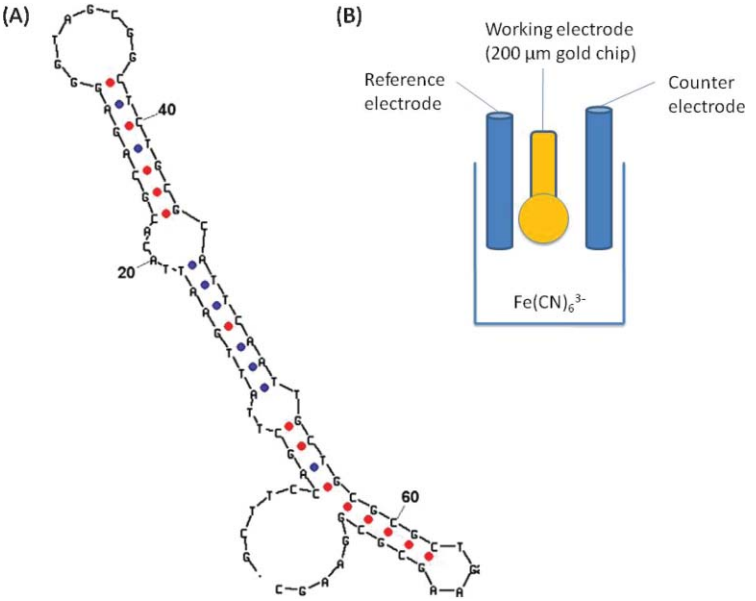

Fig. 2 (A) Secondary structure of the selected DNA aptamer used in ref. 59. (B) Electrochemical detection system and the mechanism for electrochemical signal production. An Ag/AgCl and platinum electrode was used as the reference and counter electrode, respectively. The DNA aptamer-immobilized gold electrode chip was used as a working electrode. The sample solution was tested by exposing the DNA aptamer-immobilized gold electrode to it and then inserting the chip into the sensing block containing $K_3Fe(CN)_6$. Cyclic voltammetry (CV) and square wave voltammetry (SWV) were then performed. Electron flow is generated by a redox reaction between ferrocyanide [$Fe(CN)_6^{-4}$] and ferricyanide [$Fe(CN)_6^{-3}$].

The aptamer was immobilized on gold electrodes *via* the avidin–biotin interaction, and cyclic voltammetry and square wave voltammetry were used to evaluate the target binding to the aptamer. A detection limit of 0.1 nM was achieved with good selectivity.

Moreover, some aptamers have been selected for the detection of specific pathogens; however, to the best of our knowledge, the corresponding biosensors have not been developed yet. Pan *et al.*[60] reported the direct selection of aptamers for bacterial proteins involved in bacterial invasion. In particular, they focused on *S. enterica* Serovar Typhi. Another aptamer for pathogens has been developed for *Campylobacter jejuni* by using electrophoretic analysis.[61] The selected DNA aptamer showed strong binding affinity towards *Campylobacter* with minimal cross-reactivity over other food pathogens. With the aim of detecting bacterial pathogens, a new type of assay has been conducted by using a set of 25 unique DNA sequences that bind to *Francisella tularensis*.[62] Tularemia is a very rare disease that is caused by these bacteria and humans most commonly contract this disease by handling or eating undercooked wild animal meat. A sandwich aptamer-linked immobilized sorbent assay (ALISA) has been developed with the 25-aptamer cocktail and resulted to have good sensitivity and high selectivity.

4. NA-based catalytic biosensors

The term 'nucleic acid enzyme' is used to identify nucleic acids that have catalytic activity. Ribozymes (literally enzymes made of ribonucleic acid or RNA) are found in nature and mediate phosphodiester bond cleavage and formation and peptide bond formation. In 1989, Thomas R. Cech and Sidney Altman won the Nobel Prize in chemistry for their 'discovery of catalytic properties of RNA'. Artificial ribozymes have been obtained by means of combinatorial chemistry approaches, such as *in vitro* selection and *in vitro* evolution,[63] and have been shown to catalyze quite a broad array of other chemical reactions.[63] DNAzymes are artificial molecules and are not found in nature.

4.1 DNAzymes

DNAzymes are DNA-based biocatalysts capable of performing chemical transformations.[64] These catalysts have not been found in nature, and all known DNAzymes were isolated by *in vitro* selection. So far, most of their substrates have been found to be nucleic acids. DNAzymes can therefore provide additional control over nucleic acid-based nanodevices and, because DNAzymes often catalyze multiple turnover reactions, such devices can have amplification effects. Among the many classes of DNAzymes, RNA-cleaving DNAzymes are the most widely used, mainly because of their simple reaction conditions, fast turnover rates, and significant modifications on their substrate lengths.[16]

Li and Lu were the first to employ a DNAzyme sensitive to chemical stimuli.[65] Their work has been focused on the use of the Pb^{2+}-specific DNAzyme [Fig. 3(A)]. The catalytic DNA used is '17E', a variant of the '8–17' deoxyribozyme. In the presence of the analyte the enzyme carries out a catalytic reaction such as hydrolytic cleavage [Fig. 3(B)].

The fluorosensor described in ref. 65 was constructed by labeling the 5′-end of the substrate with the fluorophore and the 3′-end of the enzyme strand with a fluorescence quencher. In the absence of lead ions, 17E exhibits a very weak activity towards the fluorogenic RNA-containing substrate. However, when lead is present, 17E rapidly cleaves the substrate, leading to a strong fluorescence signal. Lu's group has also used 17E with gold nanoparticles for the design of a colorimetric assay;[66] in this case, gold nanoparticles were coated with a 12-mer oligonucleotide [Fig. 3(C)]. In the absence of lead, the substrate hybridizes with the oligonucleotide on the particle causing aggregation of the gold nanoparticles and the production of a blue colour. In the presence of Pb^{2+}, the DNAzyme catalyzes the hydrolytic cleavage of the substrate which does not bind to the oligonucleotide on the particles any more and prevents the formation of the particle aggregate, causing the formation of a red colour. UV-Vis spectroscopy was used to detect the changes in colour and for the quantitative detection of Pb^{2+}. The interesting feature of this system is that the Pb^{2+} detection range can be modulated over several orders of magnitude by varying the relative concentrations of the DNAzyme and one of its mutant forms. With the DNAzyme only, a detection range between 100 nM and 4 mM could be obtained.

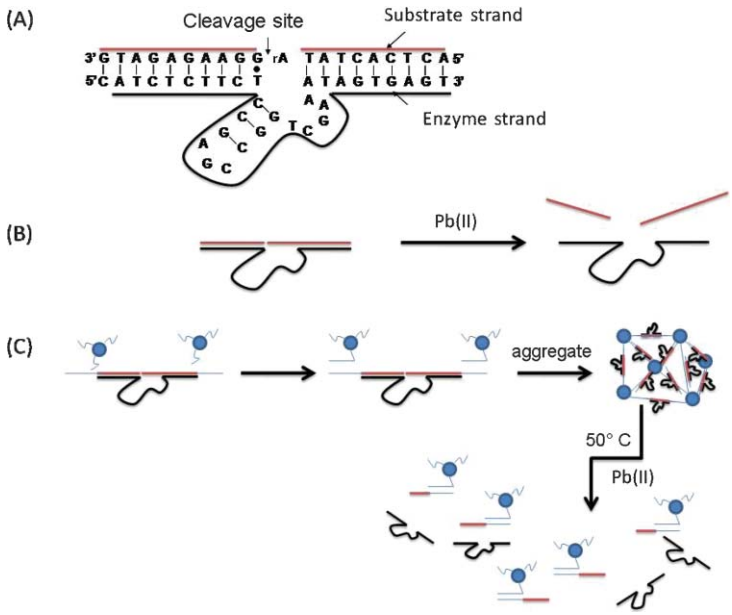

Fig. 3 (A) Secondary structure of the 8–17 DNAzyme system that consists of an enzyme strand and a substrate strand. The cleavage site is indicated by a black arrow. Except for a ribonucleoside adenosine at the cleavage site rA, all other nucleosides are deoxyribonucleosides. (B) Cleavage in the presence of Pb(II). (C) Scheme of DNAzyme-directed assembly of gold nanoparticles and their application for Pb(II) sensing. In this system the substrate stand has been extended at both 3′- and 5′-ends for 12 bases, which are complementary to the 12-mer DNA attached to the 13 nm gold nanoparticles.

The same Pb^{2+}-specific DNAzyme was also immobilized onto carbon nanotubes in collaborative works between the groups of Lu, Kane and Dordick.[67] Along the same lines of surface immobilization, the DNAzyme was covalently attached to gold surfaces[68] and gold-coated nanocapillary membranes.[69]

The Pb^{2+}-specific DNAzyme discussed above was also tested in a microfluidic device.[70,71] Micro- and nano-fluidic devices are promising platforms for several applications, and the use of DNAzymes in such devices would enable the analysis of extremely small samples, unattended long-term monitoring, and potential multiplex array sensing. In ref. 70 less than 1 nL of DNA was found to be needed to monitor the cleavage reaction for Pb^{2+} sensing. In ref. 71, the voltage-controlled microfluidic device was capable of detecting Pb^{2+} concentrations as low as 11 nM. This sensor was applied to the determination of Pb^{2+} in an electroplating sludge reference material: the results were in agreement with the certified value within 4.9%. Quantitative measurement of Pb^{2+} in this complex sample demonstrated the selectivity of this sensor scheme and points favourably to the application of such technologies to the analysis of environmental lead pollution in groundwater or drinking water.

Several other DNAzymes (or deoxyribozymes) have been selected for metal ions.[72] Seeman, Ellington and coworkers[73] recently incorporated a Cu^{2+}-dependent DNAzyme with optimized cleavage efficiency into a two-dimensional array based on the DNA double crossover motif (a rigid DNA building block frequently used in DNA nanotechnology). The array was designed to contain two types of alternating stripes: one from the DNAzyme and the other from an embedded DNA hairpin. The separation between the stripes was 32 nm. After the addition of Cu^{2+}, the DNAzyme was cleaved off the array and the separation changed to 64 nm. The authors propose that many DNAzymes responsive to different chemical stimuli could be used to construct such nanostructures, the properties of which can then be

controlled by those stimuli in a programmable manner.

4.2 Aptazymes

DNAzymes can perform chemical modifications on nucleic acids, while aptamers can bind a broad range of molecules. A combination of the two has generated a new class of functional nucleic acids known as allosteric DNAzymes or aptazymes.[16] Liu and Lu have employed an adenosine-dependent aptazyme,[74] built on the basis of the Pb^{2+}-specific DNAzyme, to assemble gold nanoparticles. In the presence of adenosine, the substrate was cleaved and the assembly inhibited. Several aptazymes (activated RNAzymes) have been shown to have metal-ion-dependent activities.[75]

In conclusion, the potentialities of aptazymes have been reported after their selection, but no direct application in environmental monitoring has been published so far.

5. NA biosensors for the detection of chemically-induced DNA damage

The elaboration of new, highly sensitive, specific, and rapid methods of determination of environmentally toxic compounds has always been a challenge to analytical chemists. This is due to the complex ecological situation and the demand for diagnosing the consequences of the impact of biologically active contaminants upon the human organism. For these reasons, the development and characterization of rapid, sensitive and inexpensive assays for the detection of chemically-induced damage to DNA caused by pollutants is an important issue in environmental monitoring. A significant number of short-term tests for genotoxicity/mutagenicity have been developed to determine the extent of environmental hazards in polluted water and sediments. These bioassays have been used in well-constructed batteries of cytotoxicity and genotoxicity tests to complement traditional approaches. These tests typically fall into one of several classes depending on their mechanism and endpoint. These classes include: bacterial mutagenesis, cultured mammalian cell mutagenesis, chromosomal damage, and DNA damage. Despite the description of short term, many of these assays are expensive to run, require sophisticated technical expertise, and are not well suited to be adapted to screening applications. In recent years, there has been an enormous increase in the use of nucleic acids as a tool in the recognition or monitoring of chemical compounds of environmental interest that may affect the genome.

In this context, DNA-based biosensors can be a valid approach to the detection of DNA damage and can be designed to be rapid, inexpensive, and user friendly.

Different kind of transducers have been employed; each of them have their proper advantages and disadvantages.

In particular, electrochemical DNA biosensors can be used: (a) for the detection of DNA strand breaks and base damage; and (b) for the detection of electroactive substances that specifically interact with DNA (covalently and/or non-covalently). The measurement of DNA damage using electrochemical biosensors has been demonstrated using the direct measurement of the oxidation–reduction properties of the bases[30,76] or indirectly using electrochemical probes.[77]

Guanine is the most easily oxidized of the four DNA bases, and has an oxidation potential of 1.06 V *versus* SCE at pH 7. The three remaining nucleobases have more positive oxidation potentials. Guanine and adenine bases in DNA can be oxidized on solid electrodes such as mercury, glassy carbon and pyrolytic graphite. Circular, covalently-closed supercoiled (sc) DNA was immobilized at a hanging mercury drop electrode (HMDE) in ref. 78. In this case the analytical signal was due to the appearance of a new ac voltammetric capacitance peak (or a constant-current chronopotentiometric Faradaic peak). These signals were caused by bases at the strand break interacting with the electrode. In these experiments, the DNA was immobilized on the electrode by a short incubation of the HMDE in about 5 μL of DNA solution, followed by washing and transfer of the modified electrode into an electrochemical cell containing supporting electrolyte.

In refs 30 and 79, disposable carbon screen-printed electrodes were used. In this approach, genomic calf thymus DNA was used as the receptor and square wave voltammetry as the detection technique. The analytical signal was the decrease of the magnitude of the oxidation current peak of guanine after interaction with the sample solution (Fig. 4).

Rusling's group showed that $Ru(bpy)_3^{2+}$ and derivatives can be used to detect DNA damage. When double-stranded (ds)DNA in ultrathin films was damaged, guanines became more available to react rapidly with the catalyst, providing an increased current in the square wave oxidation peak. $[Ru(bpy)_2PVP]^{2+}$ and $[Ru(bpy)_2PVP_{10}Cl)]Cl$, where PVP = poly(4-vinylpyridine),[80] were also used by Rusling's group to develop a self-contained, reagentless sensor for toxicity screening based on the detection of DNA damage.

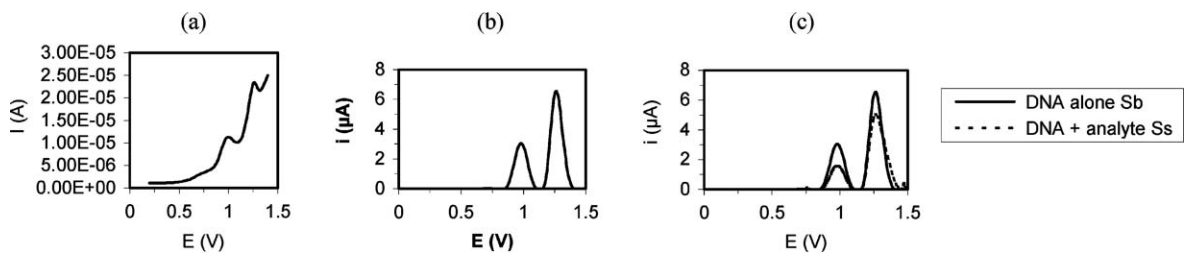

Fig. 4 Redox behaviour of guanine (+1.0 V) and adenine (+1.25 V) bases after a square wave voltammetric scan carried out with a graphite screen-printed working electrode. Shown are: the normal signal (a), the signal after a baseline correction (b), and the decrease of the DNA peaks after the interaction with a pollutant agent (c). Sb = blank signal, Ss = sample signal.

These biosensors were also used to measure toxic aromatic amines,[30] oxidative damage,[77,81,82] bioactivated benzo[*a*]-pyrene,[83] and hydrazine.[84] The damage caused to DNA by a group of ten derivatives of 1,3,5-triazine herbicides: chloro-*s*-triazines (atrazine, propazine, terbutylazine and cyanazine), thiomethyl-*s*-triazines (ametryn, prometryn, terbutryn and simetryn) and methoxy-*s*-triazines (prometon and terbumeton) was reported in ref. 85.

Also, some heavy metals are known to have great affinity for DNA and to cause mutagenesis and carcinogenesis. In 2005, Babkina and Ulakhovich,[86] proposed a method for the determination of heavy metals based on the biospecific pre-concentration of metal ions on an electrochemical DNA biosensor followed by the destruction of DNA–metal complexes with ethylenediaminetetraacetate and voltammogram recording.

DNA damage has also been measured using fluorescence-based biosensors. Time-resolved fluorescence measurements were used to detect radiation-induced changes in DNA unwinding behaviour from DNA isolated from white blood cells.[87] In another recent paper, DNA adducts of benzo[*a*]pyrene were measured using low-temperature fluorescence on a gold biosensor chip.[88] The adducts were captured using structure-specific monoclonal antibodies that were immobilized using a chemical protein linker and recombinant protein A. Detection limits for DNA adducts were in the low femtomolar range.

Changes in melting–annealing behaviour that were observed in real time using a double-strand-selective fluorescent indicator dye have also been used to measure DNA damage induced by radiation and chemical mutagens such as styrene oxide, glutaraldehyde, acrolein, allylamine, chloroacetone, acrylonitrile, bromoethane, crotonaldehyde and benzo[*a*]pyrene.[89–91] This screening assay was sensitive to various forms of DNA damage including strand breaks, cross-links and adduct formation. The assay was also demonstrated with respect to the effects of well-characterized genotoxins such as mitomycin C as well as cytotoxic but non-genotoxic compounds such as phenol, cyclohexane and toluene.

A fiber-optic capillary fluorescence system, that employs the long-wavelength intercalating fluorophore TO-PRO-3 (TP3), was used to analyze toxic aromatic amines, antibiotics, and several kinds of antitumor drugs. Compounds that interact with the TP3-DNA complex were indirectly detected by a decrease in the fluorescence intensity.[92]

DNA damage generated by organophosphate pesticides was studied by Hianik *et al.*[93] using the sensitivity of the acoustic shear wave technique to the interaction of DNA strands with damaged DNA containing apurinic or apyrimidinic (AP) sites.

6. New trends and conclusions

In summary, as reported in previous sections, NA biosensing of environmental pollution is an expanding field. Genosensors offer considerable promise for obtaining sequence-specific information in a faster, simpler and cheaper manner compared to the traditional hybridization assay. Many examples of DNA biosensors for the detection of DNA damage and interaction have also been reported. Moreover, although their appearance in the literature has been very recent, functional DNA molecules such as aptamers, DNAzymes and aptazymes have already found application in almost every aspect of DNA nanotechnology, and the resulting new materials and devices can penetrate into many other fields for practical application also including environmental monitoring.

Nanomaterials (carbon nanotubes, metal nanoparticles, or metal oxide nanostructures) will definitely have a big impact on the development of NA-based biosensors for environmental monitoring. Nanotechnology brings new possibilities for the construction of biosensors and for developing novel bioassays. Nanoscale materials have been used to promote reactions, to impose nanobarcode for biomaterials and to amplify signals of biorecognition events. Nanobiosensors were already applied in areas of cancer diagnostics, and the detection of infectious organisms, including food pathogens, and environmental analysis.[94,95]

Moreover, in the future, we are likely to see the incorporation of individual devices into more complex multifaceted systems, also called Micro Total Analysis Systems (μTAS) or Lab-on-a-chip (LOC) [a term identifying devices that integrate (multiple) laboratory functions on a single chip of only millimetres to a few square centimetres in size and that are capable of handling extremely small fluid volumes down to less than picolitres).

The integration of nanotechnology, microfluidics, and bioanalytical systems clearly represent one of the future directions of all biosensor research. Possibilities seem endless when combining these different areas, and hopes are high to solve many current problems ranging from sample preparation to real portability, from single molecule detection to true analytical speed, to reliability. Many obstacles will have to be overcome in order to fulfil all of these hopes; however, some exciting examples, typically of subsystems and not complete bioanalytical sensors, can already be found in the current literature.

For instance, a variety of microstructures have been proposed for on-chip amplification and analysis of nucleic acid, and many of them are reviewed in ref. 96. Miniaturization of PCR devices offers several advantages such as short assay time, low reagent consumption and rapid heating/cooling rates, as well as great potential of integrating multiple processing modules to reduce size and power consumption. This undoubtedly will benefit areas such as genosensor assays since multiple processes, including sample collection and pre-treatment, DNA extraction, amplification, hybridization and detection can be performed on a single, self-contained microfluidic platform.

Such miniaturization of the analytical instrumentation will enable transportation of the laboratory to the sample source (as desired for point-of-care testing).

In conclusion, in the near future, NA sensing and biosensor technology itself will undoubtedly benefit from nanotechnology and μTAS technology.

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