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DNA Diagnostics: Nanotechnology-Enhanced Electrochemical Detection of Nucleic Acids

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ABSTRACT: The detection of mismatched base pairs in DNA plays a crucial role in the diagnosis of genetic-related diseases and conditions, especially for early stage treatment. Among the various biosensors that have been used for DNA detection, EC sensors show great promise because they are capable of precise DNA recognition and efficient signal transduction. Advancements in micro- and nanotechnologies, specifically fabrication techniques and new nanomaterials, have enabled for the development of highly sensitive, highly specific sensors making them attractive for the detection of small sequence variations. Furthermore, the integration of sensors with sample preparation and fluidic processes enables for rapid, multiplexed DNA detection essential for POC clinical diagnostics. **(*Pediatr Res* 67: 458–468, 2010)**

The recent discovery and sequencing of the human genome has provided valuable insight into understanding how genetic factors contribute to the development of disease. Specifically, the detection of DNA sequence variations plays an important role in the diagnosis of genetic-related diseases and conditions, especially for early stage treatment and monitoring. Among the different types of diseases caused by DNA alterations, sequence-specific mismatch has the most importance, yet is extremely difficult to detect (1), especially for single-nucleotide polymorphism (SNP). Furthermore, sequence-specific detection has great importance in various medical and scientific applications such as the diagnosis of inherited diseases and the study of pathogen response and bacterial/viral detection.

Because of the complex nature of DNA, the detection of single or small numbers of base mismatches requires high sensitivity and specificity (2–4). Current detection methods rely on sample amplification combined with meticulous experimental stringency control (5). For example, polymerase chain reaction (PCR) requires careful primer design and accurate temperature control to obtain sensitivities in the fM range with single-base mismatch specificity (1,3,4). Although these conventional technologies provide the golden standard for laboratory-based DNA diagnostics, they cannot meet the requirements of POC clinical diagnostics (4).

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EC sensors, initially developed to detect biomolecules in a laboratory setting, have recently found extensive applications for on-site biosensing and detection (6,7), especially for medical and clinical diagnostics (8–12). While offering simplicity in operation and sample manipulation, the contemporary EC biosensor also provides highly sensitive and specific measurements for a broad spectrum of biomolecules (13–17). The sample size required for current EC sensors is small, ranging from several microliters to hundreds of nanoliters, which includes the sample pretreatment reagents. Additionally, the detection time is relatively fast, varying from a few minutes to tens of seconds. However, the most important feature of EC sensors is their potential to be easily transformed from a laboratory-based instrument to a commercializable POC device. Because of all these advantages, EC biosensing for DNA diagnostics is becoming a very promising area of research and development.

Recently, micro- and nanotechnologies have shown emerging potential in EC DNA diagnostics. EC sensors offer a perfect interface for incorporating these technologies, which includes a variety of new materials and fabrication processes. Nanomaterials can be used in various aspects of the detection system including capture probes, reporting molecules, electrode fabrication, and electrode coatings (18–25). These materials offer improved biocompatibility, additional binding sites and higher signal intensities (*via* enhanced electrical properties) compared with traditional materials in EC sensors (17,21,25–28). Nanofabrication allows for miniaturization of the sensor, which improves the sensitivity and reduces the sample and reagent volumes, making the detection process more efficient. Although nanomaterials and nanofabrication are described here as two separated categories, recent trends combine both of these elements in the design of new EC sensors for DNA diagnostics (Fig. 1). With contributions from microfluidics and MEMS technologies, EC sensors can be integrated onto portable platforms incorporating all the necessary preparation and fluidic processes (10,29,30), giving way to commercializable devices for clinical diagnostics (31,32). Ultimately, the end goal of EC sensor development is to construct a total analysis system for rapid DNA biosensing, which incorporates sample pretreatment, sample delivery, and detection.

Abbreviations: CNT, carbon nanotube; **EBL,** electron-beam lithography; **EC,** electrochemical; **EIS,** electrochemical impedance spectroscopy; **MEMS,** micro-electro-mechanical systems; **POC,** point-of-care; **SiNW,** silicon nanowire; **SNR,** signal-to-noise ratio; ***V*m**, melting potential

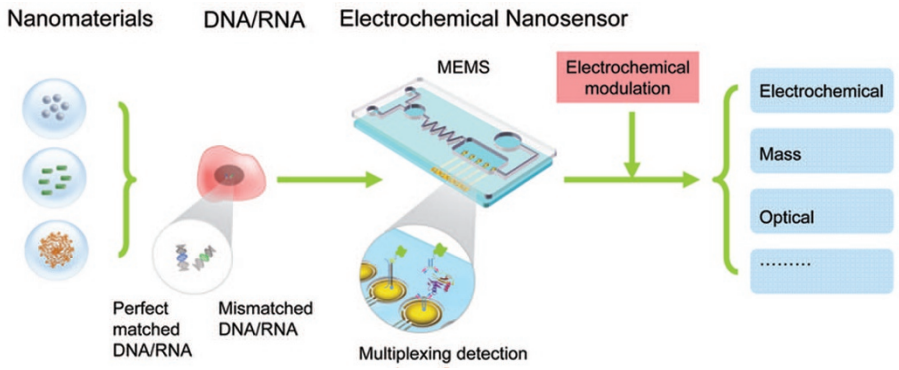

Figure 1. Schematic illustration demonstrating the integration of nanomaterials and micro/nanofabrication technologies for constructing EC DNA sensors.

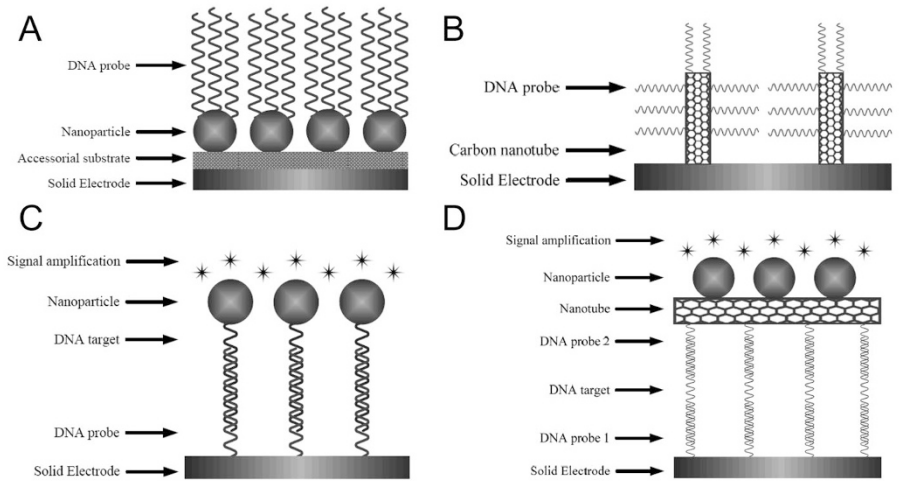

Figure 2. Common nanomaterials used in EC biosensors for DNA/RNA diagnostics; (*A*) nanomaterials for electrode coatings, (*B*) nanomaterials for probe labeling, (*C*) nanomaterials for target labeling, and (*D*) nanomaterials for signal reporting. Reprinted from Xu K *et al.* 2009 Sensors 9:5534–5557. Copyright © 2009 by authors, with permission.

Principles for EC DNA Sensors

EC detection. The EC detection of biologic species is based on EC reactions that occur during biorecognition processes (33). These reactions can be exhibited as changes of EC properties (*i.e.* current/potential, redox kinetics, impedance, *etc.*) or changes of non-EC properties (*i.e.* conformation changes, mass transportation, van der Waals interactions, *etc.*), resulting in fluctuations of an EC signal. Such fluctuations, which usually contribute to high background noise, are not sequence-specific and need to be suppressed during the detection process. The resultant signal readouts can take the form as electrical currents, potentials, or impedances in steady state or changes in these parameters during the recognition process, which correspond to the kinetics of recognition (34). Currently, *ex situ* EC sensors, in which sample pretreatment and fluidic processing are performed "off-chip," are most commonly used because they generate a better SNR, resulting from the detection of purified, concentrated biomolecules. However, these sensors have limited applications in POC diagnostics. Therefore, *in situ* EC sensors, which incorporate all the sample processing steps "on-chip," are more desirable for clinical application; however, they require higher sensitivity and specificity for non-pretreated samples. Additionally, *in situ* EC sensors can monitor changes of EC properties, which is more desirable for studying biologic processes during DNA recognition (35).

EC DNA sensors. A typical EC DNA sensor consists of an electrode, capture probe and reporter probe. A capture probe is an element used to recognize and bind to the target DNA and is usually immobilized onto a solid substrate, such as the electrode surface. However, they can also be immobilized on nanomaterials or other biomolecules. A reporter probe is a molecule that generates the EC signal in response to EC reactions. Both the capture probe and reporter probe are created with high specificity to the target DNA. Additional components, such as electrode coatings and intermediate molecular linkers, are also commonly integrated for improved sensor performance. Common molecules used as probes (capture and reporter) include single-stranded oligonucleotides, aptamers, peptides, and DNA-related proteins (14). In some sensors, the capture and reporter probes are combined together as a single unit for improved integration. Probe, target, and reporter molecules can all be modified or linked with properly integrated nanomaterials, as shown in Figure 2. Because of their high surface-to-volume ratios and biologic compatibilities, nanomaterials not only increase the signal intensity but also help to accumulate/separate specific DNA molecules during EC reactions, which greatly improves the SNR, especially for sequence-specific recognition (28). A wide variety of nanomaterials can be applied, where the most common include metal nanoparticles, cadmium sulfide nanoparticles, CNTs and SiNWs. An extensive and

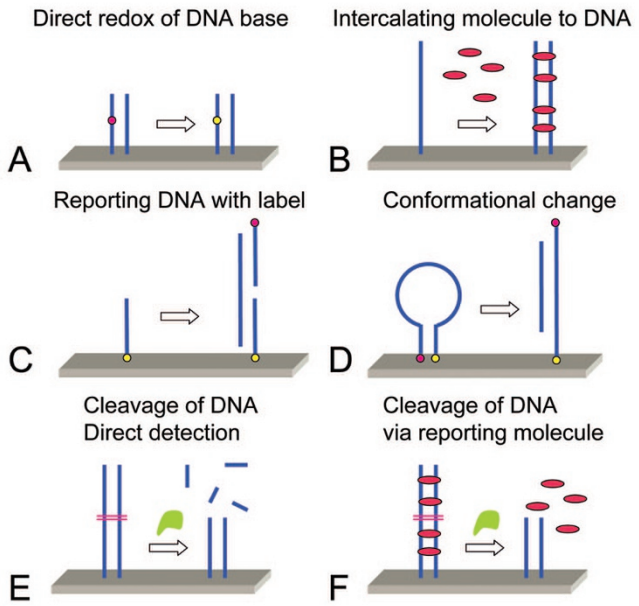

Figure 3. Schematic representation illustrating the principles for EC DNA sensors. *A*, Direct oxidation/reduction of nucleotide bases. *B*, Detection of intercalating complex for single/duplex stands. *C*, Detection of specific DNA with labeled reporting molecules. *D*, Detection of specific DNA with integrated capture probe and reporter probe. *E*, Direct detection after specific DNA enzymatic process. *F*, Detection of extra labeled reporter after specific DNA enzymatic process.

detailed review on the applications of nanomaterials for DNA biosensors can be found in literature (21,28,36).

Current EC sensors for DNA diagnostics include two schemes for biologic recognition. The most common scheme, hybridization-based DNA detection, uses nucleotides as the probe and targets elements (Fig. 3*A–D*) (37). The performance of EC sensors based on this method is highly dependent on the affinity between the probe and target molecules, which can be tuned by the probe design, environmental conditions, and additional amplification processing. This detection system can either use a one probe *versus* one target scheme, a multiple probe *versus* single target scheme, or *vice versa*. For example, EC sensors based on a sandwich detection mode are composed of one capture probe and one reporter probe for each DNA target. Ultimately, no matter what method is used for detection, the output signal is caused by changes in EC properties or EC-related properties.

The second detection scheme, which has emerged in recent years, is enzymatic-based DNA detection (Fig. 3*E* and *F*) (16,38,39). In this scheme, DNA-related enzymes are introduced into the biorecognition system and changes in amount of these enzymes correlate to specific biologic process (*i.e.* deletion/fusion of the target DNA). For example, when an EC sensor experiences a specific process, the enzyme level either increases or decreases, resulting in amplification or reduction of the signal. The enzymatic process is highly specific to a DNA sequence, which makes it ideal for DNA mismatch detection.

Advantages of EC sensors for DNA diagnostics. EC sensors offer several advantages over other detection methods, making them attractive for DNA biosensing. In addition to being highly specific and sensitive, EC sensors are extremely efficient, in terms of fast detection times and low power consumption. EC DNA sensors largely rely on nucleotide hybridization during the detection process, which involves specific electrostatic charge distributions and strong hydrogen bonding. Because the backbone of a nucleotide is composed of phosphoric acids and base units, the entire molecule is heavily charged with a negative potential. Therefore, hybridization between nucleotides needs to overcome the strong repulsion force between each other. In traditional DNA detection methods, temperature and chemicals are used to reduce the repulsion of these molecules; however, both of these modulations are not very effective. Based on the thermodynamics of DNA hybridization/denaturation, the Gibbs free energy for such processes is in the range of 1 to 10 kcal/mol. Therefore, very high temperatures or ion buffer concentrations are required to overcome this energy barrier and such conditions are likely to interfere with the bio-system. In contrast, EC sensors are capable of producing strong electrical fields, where only several hundred millivolts of potential can overcome the reaction barrier (10,40). Similar to the melting temperature (T_m) for traditional temperature control, the melting potential (V_m), which denotes the voltage at which 50% hybridization/denaturation occurs, is an important parameter useful for characterizing different DNA strands and provides an additional tool for controlling the specificity (41).

A second advantage of EC sensors is their simplicity in manipulating molecules within the sample fluid, which is performed through electrical fields generated by the electrode. Thorough mixing and precise manipulation of molecules are crucial for achieving high hybridization/denaturation efficiency. Traditional temperature or chemical-based control schemes require additional mixing and separation procedures, which greatly hinders advancement toward a POC DNA diagnostic platform. Specifically, the speed of these procedures is limited by chemical reaction times and heat/mass transfer processes within the solution. In contrast, EC sensors can generate high electrical fields within a very short time (34). By using this scheme, molecules near the electrodes can be manipulated by applying different electrical profiles. Additionally, thin dielectric double layers generated in high intensity electric fields can be used to enhance mixing and sample manipulation. Such accurate control circumvents the need for additional components and greatly simplifies the detection process, making *in situ* DNA detection possible.

A third advantage of EC sensors is their ability to achieve precise DNA recognition due to localization effects. Traditional detection methods, based on temperature or chemical control schemes, lack precision because their effects are dispersed within the entire sample solution rather than localized near the DNA molecules. In addition, the strong hydrogen bonds within DNA makes precise control over hybridization/denaturation quite difficult. In contrast, EC sensors can generate well-defined, localized electrical fields within the electrode domains where DNA recognition occurs. Additionally, nano-sized electrodes can produce electric fields that are concentrated within a small region surrounding the electrode, which allows for even greater precision and localization. Nanoelectrodes also require much smaller electrical potentials and reduces the overall power consumption of the sensor.

Because of the advantages listed above, there are several key features that EC sensors offer for DNA diagnostics:

1. Detection speed: the detection of DNA involves several processes including DNA recognition, sample manipulation, and signal readout. By using EC sensors, DNA recognition can occur within minutes and in some cases, several seconds. The entire detection process, including sample preparation, sample delivery, and signal readout, can be completed in several minutes. Such rapid detection is ideal for POC clinical diagnostics.
2. Sensitivity: with appropriate electrical field profiles, EC DNA sensors can achieve sensitivities down to several fM and in some cases, aM for short oligonucleotides. Such sensitivities can be achieved without sample amplification (*i.e.* PCR), which is a major advantage for POC systems. Additionally, the total amount of sample required for detection is on the orders of several microliters.
3. Specificity: the stringency of EC-controlled DNA recognition enables for single-base mismatch specificity, even in clinical samples.
4. Convenience: with contributions from MEMS and nanotechnology, EC sensors can be integrated onto portable platforms, enabling for POC monitoring and on-site biosensing.
5. Multiplexing: nowadays, a single biomarker is not sufficient for high specificity detection in clinical samples. By combining several biomarkers, the detection accuracy can be greatly improved, making multiplex detection for DNA diagnostics very important. Because the electrical fields generated by electrodes are highly localized, they can be used for simultaneous detection within a miniaturized platform, where the detection condition for each DNA target can be optimized separately (32,42).

EC DNA Sensor Types

Traditional EC biosensors are based on detecting changes of EC properties, such as current/potential, redox kinetics, or electrical impedance (34). However, because most biorecognition is influenced by non-EC properties (mass transportation or conformational changes) in addition to EC reactions, EC sensors can also be based on controlling the biorecognition process. From these two aspects, we will distinguish EC DNA sensors in two categories: EC biosensors as transducers and EC biosensors as controllers. In the following section, each type of sensor will be discussed in further detail.

EC sensor as a transducer. Most EC biosensors directly detect changes in EC signals, in which case, the sensor acts as a transducer. We separate EC sensors into two categories: labeled detection and label-free detection. Usually EC sensors use labeled reporters, which can result in high levels of background noise because of mass transportation processes or even conformational changes. Therefore, prelabeled reporters can help to increase the SNR. The second category, label-free detection, usually refers to detection without any sort of labeling. This scheme also includes detection which incorporates a labeled reporter in conjunction with nonlabeled targets because the final detection process is label-free.

Labeled detection. Labeling molecules are electrochemically active in that they exhibit specific EC properties, which correlate to the status of DNA targets on the electrodes. Usually, the amount of the labeling molecules indicates the amount of DNA targets in the sample either in a direct detecting mode or a competitive detecting mode (Fig. 3*C* and *F*). Nanomaterials, because of their high surface-to-volume ratios, provide more binding sites for nucleic acids. Additionally, applying nanomaterials to electrode coatings and/or labeling tags greatly improves the signal intensity. Metal and magnetic nanoparticles are commonly used because they can be easily accumulated onto the sensor surface *via* electrical or magnetic fields. By incorporating Au nanoparticles to DNA probes, Ozsoz *et al.* (24) detected Leiden mutations with a sensitivity down to 0.78 fmol. Castaneda *et al.* (25) successfully detected the single-base mismatched BRCA1 breast cancer gene and a cystic fibrosis-related gene with a combination of sandwich reactions of Au nanoparticles and magnetic beads. Using magnetic nanoparticles as labels also improves the separation efficiency, where specific DNA/RNA targets can be accumulated while removing nonspecific molecules (25,43,44).

Similar to a direct detection mode, a competitive detection mode measures changes in the output signal, which indicates increased DNA target levels. Liao and Ho (23) recently detected enterohemorrhagic *Escherichia coli* O157, a verocytotoxin (VT1/2)-producing pathogen, using a competitive EC sensor. The electrodes were modified with Au nanoparticles and a self-assembled monolayer (SAM) of thiol-capped single-stranded DNA (capture probe) for the detection of the *rfbE* gene, which is specific to *E. coli* O157. This assay is based on a competition between the target gene and reporter DNA-tagged liposomes. The sensitivity of detection for the *rfbE* gene was 0.75 aM.

In addition to detection based on changes in the amount of DNA targets, the detection process can be based on conformation changes of the labeling molecules (Fig. 3*D*). During DNA recognition, conformational change of the DNA molecule alters the distance between the labeling molecules and the electrode, which affects the EC signal. Fan *et al.* (45) first reported an ultrasensitive EC sensor based on DNA folding during hybridization. The DNA probe is designed as a hairpin structure with a ferrocene-tag at one end, which remains in a closed configuration before hybridization. After hybridizing with a complementary strand, the hairpin probe opens up, enlarging the distance between the ferrocene and the electrode, which generates a significant EC current. The sensitivity of this sensor is approximately 10 pM, even in bodily fluids (45,46). By combining this folding process with an additional amplification process, Wei *et al.* (10,11,20) detected an oral cancer mRNA gene in whole saliva. In their approach, DNA dendrimers were applied to the surface probe for improved biocompatibility and sensitivities as low as 3.9 fM of mRNA could be obtained.

Label-free detection. Label-based EC sensors require a prelabeling process, which greatly limits the variance of available reporters and the possibility for *in situ* detection. Recently, label-free EC sensors using nanomaterials have at-

tracted much attention (47,48). Although eliminating the labeling process does not significantly improve the detection efficiency, it provides the only means to realize universal *in situ* and *in vivo* DNA detection. This approach eliminates the need for additional sample separation processes or reporting molecules and allows for DNA levels to be monitored real-time within biologic systems. Label-free detection can be performed without any sort of labeling (9,13,49) or can incorporate a labeled reporter in conjunction with nonlabeled targets (10,50).

One of the simplest ways in which label-free DNA sensors operate is in the direct detection of EC reactions of nucleotide bases (Fig. 3*A* and *E*) or intercalating molecules (Fig. 3*B*). The bases in DNA have specific EC redox properties, which can be used to indicate their quantity on an electrode. However, the redox of DNA bases can be affected by other factors including the environment, hybridization events, and buffer solutions, which results in higher noise. Single/duplex-specific intercalating complexes are another type of label-free EC sensor. These intercalating molecules can either bind to the groove of a DNA duplex or insert into the planes between stacking base pairs. After the dissociation of the duplex, these molecules are released and cause a change in the EC properties. Commonly used DNA-intercalating metallic complexes include Co(2, 2′-bipyridy1)$_3^{3+}$, Co(1,10-phenanthroline)$_3^{3+}$, methylene blue, daunomycin, and aromatic amines. These compounds have been used for several clinical-related diagnostic applications, such as the detection of cystic fibrosis (51), pathogenic *E. coli* bacteria (52), *Mycobacterium tuberculosis* (52), hepatitis B virus (53), the human immunodeficiency virus (HIV) (52), and familial dysbetalipoproteinemia (54). An extensive review of this topic can be found in literature (55).

Another type of indirect, label-free EC sensor is based on a sandwich detection mode. This sensor does not require labeling of the target biomolecules; rather, only the reporting molecules are labeled (56–58). The biorecognition process is performed by monitoring changes in the amount or EC properties of the reporter molecules.

Traditional EC biosensors are based on the measurement of electrical current and potential (amperometry, voltammetry, and coulometry); however, dielectric properties can also be used for impedance-based detection (59–62). Although labeling of target molecules is not mandatory for impedance-based detection, in some cases, it can enhance the signal. Dielectric properties are highly sensitive to the conditions at the electrode interface and exhibit very unique features under certain electrical parameters such as current, potential, frequency, magnetic, and optical fields. Because EC impedance detection is a label-free method and capable of high sensitivity, it is very desirable for POC DNA sensors (49). Currently, impedance-based EC sensors can achieve sensitivities in the nM to pM range. However, obtaining such high sensitivities is still a challenging feat for impedance detection. Fundamentals of EIS and impedance-based detection can be found in literature (34,49).

Nanomaterials are important elements in impedance-based DNA sensors, especially those that poses semiconductor properties (38,59,63–68). Such materials enhance the sensor performance through increasing the electrode surface area, electrical conductivity and connectivity, chemical accessibility and electrocatalysis. The most widely used nanomaterials in impedance sensors are Au nanoparticles and CNTs, which amplify the impedance signals by forming nanoparticle-biomolecule conjugates in a solution phase. Feng *et al.* (64) reported an EIS sensor for sequence-specific DNA detection of the phosphinothricin acetyltransferase (PAT) gene. The dynamic detection range was from 1 pM to 1 μM with a detection limit of 0.3 pM.

Polymers, including chitosan and dendrimer, are another category of emerging material for impedance-based sensors (69–71). By combining CNT-Au composite nanoparticles with a polyaniline nanofiber (PAN_{nano})-carbon paste electrode (CPE), Zhou *et al.* (59) detected the PAT gene using a label-free EIS detection scheme. The dynamic range of this sensor ranged from 1.0×10^{-12} mol/L to 1.0×10^{-6} mol/L with a detection limit of 5.6×10^{-13} mol/L. Liao and Cui (61) reported a reagentless impedance biosensor for the detection of neuro-inflammatory cytokine PDGF with a limit of detection around 40 nM. Tiwari and Gong (67) developed an EC biosensor for detecting the breast cancer susceptible gene BRCA1 based on a chitosan-co-polyaniline (CHIT-co-PANI) coating on top of indium–tin-oxide. This sensor had a sensitivity of 2.1 μA/fM with an impressive response time of 16 s.

EC sensor as a controller. Traditionally, EC sensors do not interact with the biologic system during the detection process and are known as "passive mode" sensors. However, as discussed above, the detection process can be affected by the EC profile, taking into account factors such as electrolysis, electrode potential, and current. This type of sensor is known as an "active mode" sensor and acts as a controller as well as a transducer. In this scheme, the output signal is not restricted to an EC signal but can include other types of signals (*i.e.* optical, thermometric, piezoelectric, magnetic, micromechanical, and mass change), which greatly expands the application of EC sensors (72,73). Both DNA probe-target and DNA electrode interactions can be controlled by EC fields (Fig. 4). Additionally, the interactions between the electrode and DNA recognition elements can occur before, during, or after the recognition process.

Before recognition. Applications of EC fields before DNA recognition include sample accumulation (Fig. 4*C*) (74), sample separation (Fig. 4*D*) (75–78), and controlling probe surface densities (79). AC electrical fields can generate dielectrophoretic (DEP) forces for manipulating molecules within liquids, which can be applied to EC biosensing systems (80). Wang *et al.* (81) has reported an electrical focusing system for the detection of DNA/RNA at the single-molecule level by using specially-designed three-dimensional (3D) electrodes within a microfluidic reactor.

Understanding the conformation of DNA is crucial for detection in clinical samples because most model systems for EC detection use short oligonucleotides. In its natural state, DNA is in a coiled or quasi-hybridized state, taking on a complex, 3D structure. For improved hybridization efficiency, the complimentary inner/inter-structure is normally removed, so that the molecule is in a stretched conformation. In traditional DNA detection methods, high temperatures and detergent are applied to achieve

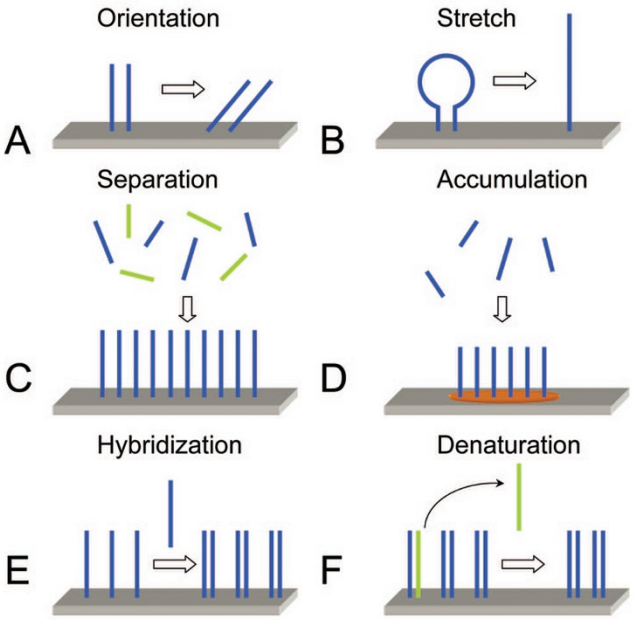

Figure 4. Schematic illustration depicting the various electrical field effects during DNA recognition, including (*A*) orientation changes, (*B*) conformational changes, (*C*) separation from interferents, (*D*) accumulation to local domain, (*E*) hybridization with complementary sequence, and (*F*) denaturation of nonspecific sequence.

this goal. By using EC sensors, this process can be accomplished by applying an electric potential, which is faster and more efficient. Within minutes, coiled DNA can be stretched out and ready for subsequent hybridization. For EC sensors with surface immobilized DNA probes, the prerecognition electrical field also arranges the probe in a more uniform angle to the surface (Fig. 4*A* and *B*) (82).

During recognition. There are numerous examples of EC field-assisted hybridization in literature (10,11,20,40,41,83,84). As previously discussed, a positive potential improves the hybridization efficiency in a short time and with a localized pattern (Fig. 4*E*) (40). In addition, the distance between the reporter and the electrode surface can also be controlled by the EC field. Based on this property, the surface of a metallic electrode can act as a quencher for the output signal. For example, in a sandwich configuration with a hairpin probe, the reporter near the electrode cannot bind with the amplifier and prevents the generation of an output signal. Only when the reporter is far from the surface it can bind with the amplifier and generate output signals (11). Analogously, optical EC sensors are based on the quenching of fluorescent signals from optical labels by the surfaces of metallic electrodes. By adjusting the EC field, the optical signal can be easily modulated. In addition, the specificity of EC DNA sensors can be guaranteed by applying the exact electrical profile corresponding to V_m of specific DNA (*i.e.* only probes and targets with the same V_m can hybridize) (41,84). Based on this concept, mismatched sequences, which have lower V_m, are differentiated from the "normal" matched sequences.

Furthermore, multiplexed detection is advantageous for the analysis of DNA and DNA-containing biomolecules (10). In this scheme, an electrode array is used where each electrode contains a specific DNA probe/target that has a different V_m, which requires a different electrical profile. This method is more precise than a temperature-based control, where the heating domain is widespread, making clearly defined boundaries difficult to achieve.

After recognition. EC fields applied after DNA recognition can transport nonspecific species away from the electrode, resulting in higher SNRs. When a positive potential is applied to accumulate DNA near the electrode, it also collects nonspecific molecules, which increases the background noise. By applying a specific negative EC field after DNA recognition, nonspecific adsorbed molecules can be removed from the electrode surface while allowing the target molecules to remain, thereby increasing the SNR (Fig. 4*F*).

Controlling the EC field during DNA recognition can be performed throughout the entire detection process and is not limited to certain stages or steps. The combinational effects of manipulating molecules and assisting DNA hybridization from EC fields results in fast and effective sensing. For example, a properly designed EC profile with turn-overs between positive and negative potentials enables for thorough mixing (11,20). The positive potential push negatively charged DNA toward the electrode whereas the negative potential cause DNA to repel. Thorough mixing before and during detection is important because it improves the reaction possibility between the probes and targets. For samples containing biomolecules other than DNA (*i.e.* peptides, proteins, *etc*.), the mixing process is mainly driven by ions inside the solution. Ultimately, appropriate EC field profiles allow for the entire recognition process to be completed within seconds with high SNRs (10,20).

Sensor Fabrication

Recent advancements in micro- and nanofabrication technologies have allowed for the development of EC DNA sensors which can precisely detect, convert, and amplify signals using various electrode configurations and nanomaterials. Adapted from the manufacturing of integrated circuits (IC) and semiconductors, surface micromachining and nanofabrication offer many advantages for DNA sensors including device miniaturization, high precision, and batch-fabrication capabilities. Additionally, microfluidics and MEMS technologies enable for sensors to be integrated into total analytical systems, allowing for rapid multiplexed detection on a portable platform for the eventual realization of POC clinical diagnostics. The following section will present an overview on technologies used in the fabrication of EC sensors, mainly for DNA biosensing applications. In-depth reviews on micro/nanofabrication technologies for EC sensors can be found in literature (85,86).

MEMS fabrication and micromachining. The fabrication of EC sensors is largely influenced by semiconductor and MEMS manufacturing due to their abilities to create high quality, high precision structures and devices. These techniques heavily rely on surface micromachining, a top-down fabrication scheme, in which a bulk material is shaped through subsequent patterning, cutting, and etching using externally controlled machines and processes. Sensors constructed using this approach are traditionally fabricated on rigid substrates

where silicon, silicon-on-insulator (SOI), and glass are the most common materials due to their compatibility with existing micromachining and nanofabrication processes. Recently, microfabricated devices are widely adapting plastics as the substrate material because they are more cost effective and suitable for batch-fabrication (87). However, plastics are not compatible with many MEMS and nanofabrication processes and therefore cannot achieve very high precision or nanoscale resolution.

Photolithography. Photolithography is one of the most fundamental processes in semiconductor and MEMS manufacturing, combining high precision patterning with batch-fabrication capabilities. Ultraviolet (UV) light passes through a mask onto a substrate, which is coated with a UV-sensitive photoresist. The light exposes the photoresist, which transfers the pattern from the mask to the substrate. The photoresist is developed in a chemical etchant, resulting in a polymer structure. This structure is commonly used as a mask for subsequent processing steps, such as metal deposition or etching, or can be used as a mold for fabricating channels, wells, and spacers for the entire sensor. Conventional MEMS-based photolithography is capable of producing features down to several microns, which is primarily dictated by the resolution of the mask and the wavelength of the UV light.

Alternative lithography. Smaller feature can be patterned through alternative lithography techniques, enabling for the fabrication of nanoelectrodes. By miniaturizing electrodes to a similar size scale as the molecules of interest (*i.e.* DNA, RNA, proteins), higher sensitivity can be achieved compared with macro-sized electrodes. Nanoelectrodes provide smaller effective surface areas for concentrating probe and target molecules, which aids in reducing the background noise. Similar to photolithography, deep UV lithography and x-ray lithography use UV lasers and x-rays, respectively, to expose the resist, which are capable of nanometer resolution. Chua *et al.* (47) fabricated SiNW arrays by first patterning the electrode fingers using x-ray lithography and performing subsequent thermal oxidation and wet etching to further define their profile (Fig. 5*B*). EBL is a mask-less approach for patterning resist, which is capable of producing feature sizes down to tens of nanometers. Rather than using light, a beam of electrons scans across the resist, exposing those regions. EBL is commonly used to pattern SiNWs electrodes (88–90), which is usually followed by thermal oxidation and wet etching. SiNW electrodes fabricated on SOI substrates enable for simplified fabrication and improved integration with semiconductor-based signal processing and communication circuits. In an alternative sensor configuration, Lee *et al.* (91) fabricated oriented nanowell (ONW) arrays within an Au electrode, which was designed so that each well could only accommodate for one or a few biomolecules (Fig. 5*C*). The nanowells were precisely patterned using EBL whereas the remainder of the electrode surface was passivated with a layer of resist, preventing nonspecific binding and enhancing the signal sensitivity. Analogous to EBL, ion-beam lithography uses a focused beam of ions to expose the resist. Such maskless approaches are relatively slow and require additional photolithographic processing for patterning larger sensor elements, such as the contact pads and electrical leads.

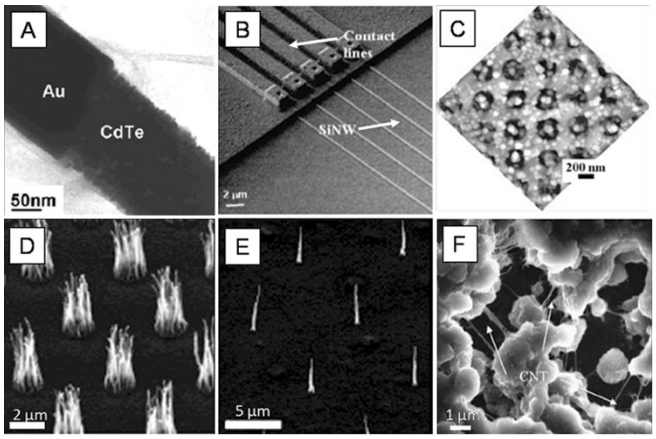

Figure 5. Images of nanomaterials and nanoelements used for EC sensors. *A*, Transmission electron microscopy (TEM) image of a CdTe-Au multi-segment nanowire. Reprinted from Wang and Ozkan, Nano Lett, 2008;8:398–404 Copyright © 2009 American Chemical Society, with permission. *B*, Scanning electron microscopy (SEM) image of patterned SiNWs, which are individually addressable by oxidepassivated metal contact lines. Reprinted from Chua *et al.,* Anal Chem, 2009;81:6266–6271 Copyright © 2009 American Chemical Society, with permission. *C*, Atomic force microscopy (AFM) image of probe ssDNA immobilized inside an ONW array. Reprinted from Lee *et al.,* Appl Phys Lett, 2006;89:113901 Copyright © 2006 American Institute of Physics, with permission. *D* and *E*, SEM images of multi-walled CNT (MWCNT) arrays patterned using UV lithography and e-beam lithography, respectively. Reprinted from Li *et al.,* Nano Lett, 2003;3:597–602 Copyright © 2003 American Chemical Society, with permission. *F*, SEM image of a CNT-poly-l-lysine film on top of a CPE. Reproduced from Jiang *et al.,* Electrochim Acta, 2008;53:2917–2924 Copyright © 2007 Elsevier Ltd., with permission.

Metal deposition. Thin-film electrodes can be fabricated by depositing metals onto a patterned resist mask through evaporation or sputtering. Metal on top of the mask is removed by selectively etching the underlying resist whereas metal that is deposited directly onto the substrate remains. Most noble metals can be patterned using this approach, making it useful for fabricating a variety of electrodes. Au is commonly used for fabricating the sensing electrode because of its high electrical conductivity, which enables for enhanced sensitivity. Additionally, Au is extremely biocompatible and its surfaces can be easily modified using SAMs, allowing for the direct immobilization of thiolated-probes (92). Electrodes that are fabricated using this approach can have thicknesses ranging from tens of nanometers to several microns, which can be precisely controlled through the deposition process. Electrodeposition, an EC process widely used for industrial metal plating, is an alternative approach more commonly used for fabricating thick-film electrodes. An electrical current is applied to an electrolyte bath containing the substrate and source material. Cations from the source material are reduced and deposited onto a conductive seed layer, which is patterned using lithography to define the shape of the electrode. Alternatively, metals can be deposited on top of screen-printed carbon electrodes (23,93) or silver wool substrates (94). A wide variety of metals can be deposited using electrodeposition (Au, Ag, Ni, Ti, Pt, *etc*.) while having the capability of producing a wide range of electrode thickness.

Nanofabrication. Electrodes fabricated using lithographic techniques are usually restricted to flat, two-dimensional (2D)

structures. Free-standing electrodes offer larger working surface areas compared with planar electrodes, which enhances the diffusion of redox species/target molecules to the surface, enabling for more pronounced hybridization signals. 3D nanoelectrodes can be constructed using bottom-up approaches, in which nano-sized components and molecules are self-guided and assembled to form the final structure. This approach can generate electrodes with highly-ordered, defect-free atomic structures, enhancing their electrical properties for higher sensitivity measurements. Free-standing nanowires can be fabricated by "nanocasting," where materials are electroplated or deposited within a mold containing nanopores. Selective etching is performed to remove the outer mold, thereby exposing the enclosed nanowires. Floating multi-segment nanowires consisting of CdTe-Au-CdTe segments were fabricated by sequential electrodeposition of metals within an alumina oxide template (Fig. 5*A*) (95). Alternatively, free-standing gold nanowires were fabricated through electroless deposition within a polycarbonate membrane followed by controlled plasma etching, allowing for the wires to remain securely embedded in the membrane (96). Nanocasting can also be used to fabricate nonmetallic nanotubes; Chang *et al.* (97) fabricated polyaniline (PANI) electrodes through polymerization within a thin nanoporous aluminum film. The alumina film was initially sputtered on top of a graphite electrode and subsequently etched in H_2S0_4, resulting in vertically oriented PANI nanotubes arrays.

Recently, CNTs have shown great potential as an electrode material for EC DNA detection due to its superior mechanical and electrical properties (98,99). Specifically, CNTs demonstrate rapid electron transport, amplifying the detection signal and making them effective transducers. Additionally, the well-defined chemistry of CNTs allows for precise immobilization of probe molecules by adsorption or chemical grafting. CNTs can be grown using several processes, including arc discharge, laser ablation, and chemical vapor deposition (CVD). Of these, plasma-enhanced CVD is the most common method for fabricating CNT electrodes due to its ability to precisely pattern nanotubes with specific orientations (Fig. 5*D* and *E*). The growth of CNTs is initiated by metal nanostructure catalysts (Ni, Co, or Fe), which are typically patterned *via* lithography. This technique commonly results in a bundled, forest-like CNT configuration (Fig. 5*D*) (100,101), which increases the effective surface area; however, lacks the spatial resolution for single molecule detection (102). Improved sensitivity can be achieved by embedding CNT arrays within a SiO_2 matrix, which enhances mechanical stability and electrical isolation of the electrodes (66,103,104).

Printing. Screen printing is a thick-film patterning/deposition technique capable of large-scale sensor production. Screen printed electrodes (SPEs) have been used for various EC DNA sensors due to their straightforward fabrication, high uniformity, and material versatility (105,106). A paste or ink is spread over an emulsion or steel screen, containing the electrode pattern. The paste is usually a mixture of an organic binder, a solvent and the electrode material, which commonly are metallic (Au, Pt, Ag), ceramic (Al_2O_3, ZrO_2), or carbon nanoparticles. The pattern is transferred onto the substrate by forcing the paste through the screen's openings. The paste is then set to dry, removing the solvents, followed by firing to burn off the organic binder. Alternatively, inks can be deposited using a printer for enhanced automation and precision (96,107,108). EC sensors commonly use CPEs composed of carbon nanomaterials (*i.e.* graphite, carbon fibers, CNTs), which does not require high-temperature processing, allowing them to be fabricated on plastic substrates. Screen printing can produce electrodes with thicknesses of several millimeters with minimum features of $\sim$100 μm, without the use of expensive equipment or a clean room facility.

Surface chemical property modification. Recent advancements in surface modification technologies have led to significant improvements in sensor performance. EC sensors commonly use multilayer electrodes, which consist of thin layers of polymers, nanoparticles, or nanoparticle-polymer composites stacked on top of the electrode. Such films can enhance the sensitivity and specificity of the sensor by acting as a 3D matrix for entrapping nucleotide probes and reducing the interference from nonspecific molecules, which can contribute to background noise. Additionally, conductive coatings can enhance the signal of redox species and minimize the loss of signal from the electrode to the electrical circuitry. Because of their high electrical conductivity and enlarged surface area, carbon and metallic nanoparticles are commonly used for particle-based coatings (59,64,109–111). In this approach, a solution containing nanoparticles is dispensed on the electrode surface and allowed to dry. As the solvent evaporates, the nanoparticles form SAMs, which are held together through intermolecular interactions (*i.e.* Van der Waals, electrostatic, *etc*.).

Alternatively, nanoparticles can be incorporated within polymer films, which allows for the particles to remain securely embedded within rigid matrix for enhanced robustness. Nanoparticles are commonly integrated into the polymer solution before polymerization, which can be initialized by heat, light, plasma, or electrical current. A CNT-poly-l-lysine film was fabricated by depositing a layer of CNTs on top of a CPE followed by application of poly-l-lysine solution and subsequent electro-polymerizing (Fig. 5*F*) (112). Additionally, conductive polymers [*i.e.* polypyrrol, polypyrrol propylic acid (PPA), PAN] can also be used as electrode coatings for signal enhancement (60,64,67,72,113). By applying pulsed electric fields, polymers can be locally electro-polymerized directly on the electrode surface, without the need for additional patterning procedures. Nonconductive polymer films, such as polyamidoamine dendrimers, can be deposited through various chemical bonding schemes (69,71).

Prospects

The future direction of EC DNA sensors is focused on the development of POC systems, which seek to integrate sample handling, fluidic processing, and detection on a portable platform. Although EC biosensors have been widely developed for laboratory-based detection within the past several years, there are very few successful POC devices for clinical diagnostics that are currently commercialized (*i.e.* glucometers).

Clinical applications for EC DNA sensors are still far from reality due to several important issues. Although much work has been done to improve the performance of EC DNA sensors, the sensitivity/specificity is still a key issue. Specifically, the detection of clinical samples requires high sensitivity/specificity as well as high repeatability/reliability, which is still an unresolved problem. To address these issues, new nanomaterials with effective and stable performance are required along with higher stringency control during manufacturing. Additionally, the accuracy for clinical detection can be enhanced though bio-statistic support based on multiple DNA biomarkers. To improve the application of EC sensors for real clinical tests, a simple detection process is desired, which incorporates automatic sample processing or *in situ* detection. This can be achieved through using micro/nanotechnologies, which offers new materials and sensor fabrication processes. Furthermore, the safety of nanomaterials is becoming a significant issue, especially as applications for these materials become more widespread.

Sensitivity and specificity. Current EC DNA sensors require labeling to achieve high sensitivity and specificity. Although label-free technology provides convenience for low cost and *in situ* detection, it suffers from high noise and false positives, which pose a serious problem for achieving high specificity. In most cases, signal amplification is necessary and in particular, specific-signal amplification is required for achieving a good SNR (10). Alternatively, new nanomaterials with DNA-specific binding properties can enhance signal amplification and improve overall sensor performance (114–116).

Repeatability and reliability. The ability to produce repeatable and highly reliable measurements is one of the most important challenges facing EC DNA sensors, especially for clinical diagnostics and commercial usage. Clinical samples are prone to a high degree of variability, which results from physiologic and lifestyle differences between patients. Additionally, slight variations in the actual sensor (*i.e.* electrode geometry, uniformity of coatings and probes, *etc*.) can lead to inconsistencies in measurements, particularly for the detection of small sequence variations. In addition to improving the detection sensitivity and specificity through the use of nanomaterials and nanoelectrodes, optimized fabrication processes and higher stringency control during manufacturing can enhance the overall repeatability and reliability of the sensor.

Biostatistical support. The performance of EC DNA sensors is largely dependent on the accuracy of the targeting biomarker(s). However, DNA biomarkers for clinical diagnostics still lack the accuracy needed for highly specific detection. Because of the complexity and nonlinearity of the human body, sequence mutations for single DNA/RNA do not necessarily correspond to one specific disease. Recently, a panel of multiple biomarkers resulted in improved accuracy for clinical diagnostics (10,117,118). Therefore, multiplexed detection will be very dominant in the future development of EC nanosensors for sequence-specific detection.

Sample pretreatment. Clinical samples are complex mixtures, which contain a multitude of components and biologic species. Even *in vitro* detection of body fluids (*i.e.* blood, urine, and saliva) presents great challenges for simple detection systems. Usually, several pretreatment processes are required before detection, such as separation, purification, accumulation, and amplification. Application of nanomaterials can greatly simplify and improve the efficiency of such pretreatment processes. Additionally, MEMS and nanofabrication technologies enable for the construction of portable, automated devices with batch-fabrication capabilities. Current research is focused on total system integration where several promising devices have already been demonstrated (10,12,119,120).

Toxicity of nanomaterials. Although EC DNA sensor performance is greatly enhanced by nanomaterials, their cross-linked structures and associated organic reagents can present serious toxicity problems in biomedical systems (121,122). Therefore, new nanomaterials with low toxicity are in high demand. Recently, new biocompatible nanomaterials have been developed (123–125), including ones which contain biologic backbones, which have attracted extensive attention (20,126,127).

CONCLUSIONS

EC sensors show great potential for DNA biosensing, offering high sensitivity and specificity essential for single-base mismatch detection. Advancements in micro/nanotechnologies, specifically fabrication techniques and new nanomaterials, are largely responsible for improvements in EC sensors. In particular, the detection sensitivity is enhanced through highly-specific molecular recognition (by appropriately-designed targets and probes), improved EC signal generation, transduction and amplification, and enhanced electrical conductivity for minimized background noise. Additionally, EC sensors are extremely efficient, in terms of fast detection times, low power consumption, and electrode multifunctionality (*i.e.* sample manipulation, polymer electropolymerization, and DNA detection). Contributions from microfluidics and MEMS fabrication allow for EC sensors to be integrated with relevant sample handling and fluidic processes on a portable diagnostic platform, which enables for rapid, multiplexed, and high throughput analysis. With further development and integration of emerging technologies, EC DNA sensors will become more prominent clinical diagnostic tools for detecting a broad spectrum of genetic-related diseases and conditions.

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