Chip-Based Sensors for Disease Diagnosis

by

Zhichao Fang

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Pharmaceutical Sciences
University of Toronto

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Pharmaceutical Sciences
University of Toronto
2010

Abstract

Nucleic acid analysis is one of the most important disease diagnostic approaches in medical practice, and has been commonly used in cancer biomarker detection, bacterial speciation and many other fields in laboratory. Currently, the application of powerful research methods for genetic analysis, including the polymerase chain reaction (PCR), DNA sequencing, and gene expression profiling using fluorescence microarrays, are not widely used in hospitals and extended-care units due to high-cost, long detection times, and extensive sample preparation. Bioassays, especially chip-based electrochemical sensors, may be suitable for the next generation of rapid, sensitive, and multiplexed detection tools.

Herein, we report three different microelectrode platforms with capabilities enabled by nano- and microtechnology: nanoelectrode ensembles (NEEs), nanostructured microelectrodes (NMEs), and hierarchical nanostructured microelectrodes (HNMEs), all of which are able to directly detect unpurified RNA in clinical samples without enzymatic amplification. Biomarkers that are cancer and infectious disease relevant to clinical medicine were chosen to be the targets. Markers were successfully detected with clinically-relevant sensitivity. Using peptide nucleic acids (PNAs) as probes and an electrocatalytic reporter system, NEEs were able to detect prostate cancer-related gene fusions in tumor tissue samples with 100 ng of RNA. The development of
NMEs improved the sensitivity of the assay further to 10 aM of DNA target, and multiplexed detection of RNA sequences of different prostate cancer-related gene fusion types was achieved on the chip-based NMEs platform. An HNMEs chip integrated with a bacterial lysis device was able to detect as few as 25 cfu bacteria in 30 minutes and monitor the detection in real time. Bacterial detection could also be performed in neat urine samples. The development of these versatile clinical diagnostic tools could be extended to the detection of various cancers, genetic, and infectious diseases.


Acknowledgements

During my 5-year Ph.D. program, many people have given me great support. The most important ones are my supervisor Prof. Shana O. Kelley and Prof. Edward H. Sargent. Their wise counsel and intellectual advice inspire the whole team to explore our projects one step further, and from one victory to another. No matter if our research is soaring in the sky or struggling in difficulties, they are always confident and encouraging. From Boston to Toronto, I have watched our biosensing chip from a preliminary device to the present automated integrated circuit. During the growth, it encountered various problems, but every time we presented discouraging results, the feedback from Prof. Kelley and Prof. Sargent has always been positive and helpful, and their suggestions always enlighten the correct path to a solution. Studying in their laboratories is a pleasure and wonderful experience, not to mention having sufficient funding and instrumentation. I also received important advices from other professors, including Prof. Axel Guenther, Prof. Rob Macgregor, Dr. Jeremy A. Squire, Prof. Yu Sun, Prof. Gilbert Walker, and Prof. David R. Walt.

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My undergraduate advisor, Prof. Xin Sheng Zhao, and laboratory members, Dr. Su Guo, Dr. Wei Liao, and Dr. Fang Wei, in Peking University, showed me the correct attitude towards science and trained me well for studying abroad.

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Chapter 1
Introduction

1 Medical diagnosis

There is no more important field in medicine than diagnosis. Without it, we are charlatans or witch doctors treating in the dark with potions and prayers.

Paul Cutler (Cutler, 1979)

In the health care system, medical diagnosis and therapy are the two major parts. As the step prior to treatment, medical diagnosis provides identification and staging for a medical condition or disease, and this determines the appropriate therapeutic procedure. No treatment can be delivered without reliable diagnosis, and the success of the therapy depends on the accuracy of diagnosis.

1.1 Classification and development of medical diagnosis

Generally, three types of diagnostic methods can be applied according to the disease state of the individual patient: i) symptomatic diagnosis, which recognizes the outward signals and symptoms of the disease or condition, ii) physiological/biochemical diagnosis, which uses clinical tests to analyze the samples taken from a patient, and iii) genetic analysis, which examines the disorder of the gene sequences of the patient (Table 1.1).
Table 1.1. Comparison of medical diagnosis methods

<table>
<thead>
<tr>
<th></th>
<th>Symptomatic diagnosis</th>
<th>Physiological/Biochemical diagnosis</th>
<th>Genetic analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Objectives</strong></td>
<td>- Blood pressure</td>
<td>- Glucose blood level</td>
<td>- DNA/RNA sequence</td>
</tr>
<tr>
<td></td>
<td>- Pulse rate</td>
<td>- PSA level</td>
<td>- Biosensing</td>
</tr>
<tr>
<td><strong>Methods</strong></td>
<td>- Sphygmomanometer</td>
<td>- Electrochemistry</td>
<td>- PCR</td>
</tr>
<tr>
<td></td>
<td>- Stethoscope</td>
<td>- Enzymatic reaction</td>
<td>- DNA sequencing</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Microarray</td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
<td>Direct</td>
<td>Accurate</td>
<td>Fundamental</td>
</tr>
<tr>
<td></td>
<td>Rapid</td>
<td>Reliable (if mechanism is clear)</td>
<td>Accurate</td>
</tr>
<tr>
<td></td>
<td>Low cost</td>
<td>More information</td>
<td>Personalized</td>
</tr>
<tr>
<td></td>
<td>Easy to conduct</td>
<td></td>
<td>Diagnostic &amp; prognostic</td>
</tr>
<tr>
<td><strong>Limitations</strong></td>
<td>Limited information</td>
<td>Time consuming</td>
<td>Time consuming</td>
</tr>
<tr>
<td></td>
<td>Inaccurate</td>
<td>Expensive</td>
<td>Expensive</td>
</tr>
<tr>
<td></td>
<td>Unreliable</td>
<td>Expertise &amp; training</td>
<td>Expertise &amp; training</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Insufficient detection limit</td>
<td>Insufficient detection limit</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The development of symptomatic diagnosis by physical examination, which is the most straightforward and easy-to-conduct method, began in earnest from the days of ancient China, Egypt, and Greece. In Traditional Chinese Medicine (TCM), the four types of diagnostic methods commonly used by doctors are: inspection, auscultation-olfaction, interrogation, and palpation. However, these methods heavily rely on experience and require considerable diagnostic skills, which are gained over many years even decades of training in order to understand the full complexity of symptoms and dynamic balances. In today’s medical diagnosis, more techniques and tools are available to assist doctors to diagnose based on the symptoms of the patient. However, symptomatic diagnosis, such as measuring blood pressure and heart rate, still serves as the preliminary step, since these methods provide basic and rapid information. Nevertheless, the information is insufficient to make a diagnosis in most cases.

As the development of biotechnology continues, more sophisticated tests, such as medical imaging, urine analysis, and blood test, became available for doctors. By measuring the presence, concentration or activity of clinically relevant targets, doctors were able to diagnose the disease more accurately and measure the progress or recovery from the disease. However, these methods also have some limitations. For instance, some of them are time-consuming and expensive, and in order to perform the test and interpret the data, special expertise and training are required.
Moreover, the sensitivity and specificity of some methods are not sufficient to detect disease markers that are often present at very low concentration levels.

Since the late 20th century, especially with the completion of the human genome sequencing project, the understanding of disease mechanisms has been greatly improved. The wealth of information about the mutations of the human genes associated with different disease states have provided researchers with many markers for detection of the diseases. For instance, the initial dose of warfarin, the most widely prescribed oral anticoagulant drug in North America and Europe, can be determined more accurately with patient genotyping. The mutation of CYP2C9 gene is responsible for reducing warfarin metabolism both in vitro and in vivo (Furuya et al., 1995; King et al., 2004; Sanderson et al., 2005). Individual responses to warfarin vary and can lead to overdosing or underdosing, resulting in bleeding or blood clotting, respectively. Regular physiological and biochemical tests fail to predict the correct dosing, so warfarin-related drug events have been the leading cause for emergency room visits. Genotyping patients in order to detect CYP2C9 mutations could therefore prevent this type of adverse reaction.

Genetic analysis is therefore a powerful tool for disease diagnosis and prognosis. However, the conventional genetic analysis methods, such as polymerase chain reaction (PCR) and DNA sequencing, are not cost effective and are very time consuming. In order to detect warfarin dosing related genotypes, existing biochemical genetic testing methods, including PCR and sequencing, are slow (approximately a day) and costly (approximately $350 per single test). Therefore, genetic tests are only recommended by FDA, but are not required. In addition, special expertise and training are also needed to analyze the data and interpret the results. All of these requirements result in high-cost diagnostics (Table 1.2).
<table>
<thead>
<tr>
<th>Test (company)</th>
<th>Application</th>
<th>Technology</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlloMap (XDx)</td>
<td>Predicts whether transplant patients is rejecting the donor’s heart</td>
<td>Analyzes expression of 20 genes from blood sample</td>
<td>$2,950</td>
</tr>
<tr>
<td>AmpliChip CYP450 (Roche Diagnostics)</td>
<td>Tests cytochrome P450 genes to identify alleles associated with fast or slow drug metabolism</td>
<td>Genotypes sequences with microarrays</td>
<td>$500</td>
</tr>
<tr>
<td>BRAC Analysis (Myriad Genetics)</td>
<td>Assesses risk of breast cancer and ovarian cancer in patients with a family history of cancer</td>
<td>Analyzes DNA to detect mutations in BRCA1 and BRCA2 genes</td>
<td>$2,975 (for most extensive version)</td>
</tr>
<tr>
<td>ChemoFx (Precision Therapeutics)</td>
<td>Quantifies an individual patient’s likely response to cancer drugs</td>
<td>Cultures tumor cells and exposes to drugs; evaluates immunohistochemically</td>
<td>$450 for each drug tested</td>
</tr>
<tr>
<td>Familion (Clinical Data)</td>
<td>Assesses disease-causing potential of genes associated with cardiac channelopathies (long QT and Brugada syndromes)</td>
<td>Sequences five cardiac ionic channel genes from a blood sample (KCNQ1, KCNH2, SCN5A, KCNE1, KCNE2)</td>
<td>$5,400 (for most extensive version)</td>
</tr>
<tr>
<td>MammaPrint (Agendia)</td>
<td>Predicts risk of recurrence in node-negative breast cancer after surgery</td>
<td>Analyzes expression of 70 genes in frozen tissue using microarrays</td>
<td>&gt;$3,000</td>
</tr>
<tr>
<td>Oncotype DX (Genomic Health)</td>
<td>Predicts risk of recurrence in node-negative, hormone-sensitive breast cancer after surgery</td>
<td>Analyzes expression of 21 genes in tumor sample using RT-PCR</td>
<td>$3,460</td>
</tr>
<tr>
<td>Phenosense GT (Monogram Biosciences)</td>
<td>Identifies most effective HIV drugs for infected patients</td>
<td>Uses key HIV genes to create vector that is tested for drug susceptibility</td>
<td>$1,460</td>
</tr>
<tr>
<td>PreGen-Plus (Exact Sciences)</td>
<td>Sold through LabCorp; stool-based DNA screening assay for colorectal cancer</td>
<td>Analyzes DNA in stool for point mutations in 3 genes and for microsatellite instability and sign of apoptosis</td>
<td>$500</td>
</tr>
<tr>
<td>Prostate PX (Aureon)</td>
<td>Predicts risk of recurrence in prostate cancer after prostatectomy</td>
<td>Assesses 8 markers, including clinical factors and distribution of key proteins in cells</td>
<td>$1,968</td>
</tr>
</tbody>
</table>

(Table adapted from Baker, 2006.)
1.2 Prostate cancer diagnosis

Cancer diagnosis and hospital-acquired bacterial speciation are serious health problems in the world that need better diagnostic tools urgently.

Among Canadian men, prostate cancer is the most commonly diagnosed cancer. It is estimated that in 2010, about 24,600 men will be diagnosed with prostate cancer and 4,300 will die. A man’s life time risk of prostate cancer is 1 in 7 (www.cancer.ca).

During the asymptomatic stage, prostate cancer can often be detected quickly and easily using the prostate-specific antigen (PSA) blood test (www.prostatecancerfoundation.org). PSA is a protein produced by the prostate and released in very small amounts into the bloodstream. The accepted normal level of PSA in blood is under 4 ng/mL. If the prostate becomes enlarged, more PSA is released. Levels of 10 ng/mL or over PSA in blood usually suggest a serious problem. However, a high level of PSA alone does not confirm prostate cancer. High PSA level can be due to other prostate problems, such as benign prostatic hyperplasia (BPH) or prostatitis. Moreover, some prostate cancer cases with low levels of PSA have also been reported (Carter, 2004).

Certain genetic factors have been shown to play a vital role in the development of prostate cancer (Tomlins et al., 2005; Cerveira et al., 2006; Perner et al., 2006; Soller et al., 2006; Wang et al., 2006b; Nam et al., 2007). In prostate cancer cell lines, recurrent gene fusions, or DNA rearrangements were commonly found. For instance, in 2005 Timlins and coworkers reported in Science that recurrent gene fusions of the 5’ untranslated region of TMPRSS2 to ERG or ETV1 were prevalent in prostate cancer tissues. The position and sequences of the fusion points were identified and illustrated in Figure 1.1 (Tomlins et al., 2005). Researchers have also confirmed that the gene fusions in prostate cancer cell lines and the TMPRSS2:ERG gene fusion could be found in more than 50% of prostate cancer samples (Tomlins et al., 2005; Cerveira et al., 2006; Perner et al., 2006; Soller et al., 2006; Wang et al., 2006b; Nam et al., 2007). Ittmann and coworkers discovered several other types of TMPRSS2:ERG fusions (Wang et al., 2006b). One of the TMPRSS2:ERG fusions, in which the exon 1 of TMPRSS2 (1-71 bp) fused to the exon 4 of ERG (226-762 bp), had the highest recurrence in prostate cancer cells (Tomlins et al., 2005; Wang et al., 2006b).
Figure 1.1. Schematic illustration of prostate cancer-related \textit{TMPRSS2:ERG} gene fusion (Tomlins et al., 2005).

\textit{TMPRSS2:ERG} fusion is not only a diagnostic factor but also a prognostic factor. Patients who had tumors with \textit{TMPRSS2:ERG} gene fusion had a much greater rate of cancer recurrence in 5 years (58.4\%) than patients who lacked the fusion gene (8.1\%) (Nam \textit{et al.}, 2007). Therefore, these patients still need special attention after the cancer removal surgery.

Currently, these gene fusions are detected using advanced biochemical methods, such as fluorescence \textit{in situ} hybridization (FISH), or real-time polymerase chain reaction (qPCR). However, these tests are expensive, time-consuming and can have either poor sensitivity or poor reliability. Considering the advantages of electrochemistry, detection of these gene fusions by electroanalytical methods would offer a sensitive, reliable, and low cost alternative in analyzing clinical samples.

1.3 Bacterial speciation

Bacterial infections cause thousands of deaths and millions of dollars lost around the world annually. It is estimated that 5-10\% of all hospital patients acquire some type of nosocomial infection (Tortora \textit{et al.}, 2002). Currently, the classification and identification of unknown bacteria are performed using the criteria and methods listed in Table 1.3.
Table 1.3. Taxonomic criteria and methods for classifying and identifying bacteria

<table>
<thead>
<tr>
<th>Criterion or Method</th>
<th>Classification</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphological characteristics</td>
<td>No (yes for cyanobacteria)</td>
<td>Yes</td>
</tr>
<tr>
<td>Differential staining</td>
<td>Yes (for cell wall type)</td>
<td>Yes</td>
</tr>
<tr>
<td>Biochemical testing</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Serology</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Phage typing</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Fatty acid profiles</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Protein analysis</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>DNA base composition</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>DNA fingerprinting</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>rRNA sequencing</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>PCR</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Nucleic acid hybridization</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

(Postlethwait and Hopson, 1995; Black, 1996; Tortora et al., 2002)

Morphological characteristics and differential staining can only identify microorganisms by their structures and cell wall staining results, respectively. While these tests are quick, many microorganisms look too similar for an accurate diagnosis. Biochemical testing, serology, and phage typing are required. These tests can give a definite answer. The major drawback is the length of time, which usually takes one or more days. In order to shorten the time for testing,
some rapid identification methods have been developed. For instance, the Enterotube™ II from Becton Dickinson can perform 12 different biochemical tests simultaneously (Hayek and Willis, 1984). However, it still needs to be incubated at 37 °C for 24 hours. In addition, the color result for positive and negative is hard to distinguish for some reactions, so it might yield false positive or false negative results (Figure 1.2B).

![BBL Enterotube™ II](image)

**Figure 1.2.** (A) Schematic illustration of positive and negative results of Enterotube™ II from Becton Dickinson. (B) Real image of Enterotube™ II after bacterial incubation. (Figure adapted from www.msu.edu.)

DNA analysis methods, such as PCR and nucleic acid hybridization, could both classify and identify bacteria. However, because of the insufficient sensitivity and specificity of these techniques, bacterial culturing is required, and this takes at least 24 hours, making it the rate-determining step. Biosensing devices could serve as alternatives to achieve sensitive and cost-effective way to detect genomic sequences in clinical samples.
2 Bioassays

Bioassays convert biological reactions into physicochemical readouts and are important tools for clinical diagnostics and the biological sciences.

2.1 Classification of bioassays

There are different ways to classify bioassays. With respect to the molecular recognition mechanisms, they could be classified as listed in Table 1.4 (Scheller et al., 2001).
Table 1.4. Bioassay classification with respect to molecular recognition

<table>
<thead>
<tr>
<th>Principles</th>
<th>Type of probes</th>
<th>Examples and references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic reaction</td>
<td>Enzymes</td>
<td>Blood glucose sensors (Meyerhoff et al., 1999; Tierney et al., 1999; Chen et al., 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibody-antigen interaction</td>
<td>Antibodies</td>
<td>Detection of human IgG using goat anti-human IgG (DeLisa et al., 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antibody analogs</td>
<td>Detection of thrombin using aptamer (Wang et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agonist-receptor interaction</td>
<td>Receptors</td>
<td>Detection of G protein activation using G protein-coupled receptors (Bieri et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Watson-Crick base pairing rules</td>
<td>Nucleic acids</td>
<td>Detection of genomic sequences using DNA probes (Drummond et al., 2003; Lapierre et al., 2003; Gasparac et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Nucleic acid analogs</td>
<td>Detection of genomic sequences using PNA probes (Yao et al., 2004; Fang and Kelley, 2009; Soleymani et al., 2009a)</td>
</tr>
<tr>
<td>Other interactions</td>
<td>Bacteriophages</td>
<td>Detection of bacteria (Shabani et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Molecularly imprinted polymers</td>
<td>Detection of proteins (Bossi et al., 2001)</td>
</tr>
</tbody>
</table>

2.2 Nucleic acid bioassays

Among these bioassays, detection of nucleic acids, such as DNA and RNA, is especially well studied because the Watson-Crick base pairing interactions between complementary strands are specific and robust.
Typical nucleic acid sensors are chip-based. A single-stranded probe is immobilized on a solid surface, such as Au or glass slides, and forms a self-assemble monolayer. When the target sequence strands are introduced onto the recognition layer of probe, the complementary strands are captured according to the Watson-Crick base pairing rules, and form the stable duplex structure (Figure 1.3). Various reporting methods could be used to detect signals before and after the hybridization event to monitor the recognition. Mass readout, optical readout, and electrochemical readout are commonly used.

Figure 1.3. Schematic illustration of general nucleic acid sensor design. Probe molecules are immobilized on the solid surface and able to capture the complementary target nucleic acids. The resulting hybridization is reported via various methods of signal transduction.

2.2.1 Mass readout

One of the straightforward changes after target hybridization is the difference in mass, which is often monitored by a quartz crystal microbalance (QCM) (Caruso et al., 1997; Towery et al., 2001; Mannelli et al., 2003). Combined with other technologies, QCM can detect DNA targets at sub-nanomolar range (Patolsky et al., 2001; Wu et al., 2007) and point mutations (Ye et al., 2009).

Due to the multiple steps to eliminate the background noise level and non-specific adsorption, the time required for each measurement is unacceptable for rapid detection. So far, the QCM has not been widely used in development of multiplexed chip-based sensors.
2.2.2 Optical readout

An alternative readout method is to monitor the optical signals before and after target hybridization. Detection of fluorescent signals is one of the most developed strategies, which is extensively used in nucleic acid detection and microarrays (Epstein et al., 2002; Lee and Whitmore, 2002; Simon et al., 2002; Yang and Speed, 2002; Blalock et al., 2003; Guo, 2003; Kerr, 2003; Glonek and Solomon, 2004; Schotter et al., 2004; Jørstad et al., 2007; Sassolas et al., 2008; Russell et al., 2009). Fluorescence-based techniques could reach high sensitivity and specificity, by which single-molecule detection and single-nucleotide polymorphism (SNP) discrimination could be achieved (Gunnarsson et al., 2008; Xiao et al., 2009).

Fluorescence methods have several limitations. A well-known limitation is photobleaching. Fluorophores in an excited singlet state can undergo a covalent modification procedure that destroys their ability to emit fluorescence, resulting in a loss of fluorescent signals. Secondly, the labeling of fluorescent dye can be time-consuming and has effects on the physiochemical behavior and the concentration levels of the target molecules.

Another strategy is to use surface plasmon resonance (SPR) to measure adsorption of analytes onto planar metal surface. The label-free technique of SPR is an excellent candidate for detection of DNA or RNA at low concentrations directly, with its femtomolar sensitivity after signal amplification (Chen et al., 2004; Goodrich et al., 2004). However, the high-cost of the instruments and difficulties in multiplexed detection limit the application of SPR in bioassays.

2.2.3 Electrochemical readout

Compared to QCM or SPR, which requires costly instrumentation and fluorescence-related methods, which are associated with photobleaching effects and time-consuming labeling steps, electrochemistry offers a cost-effective way for nucleic acid biosensing (Drummond et al., 2003). Electrochemical signals can be obtained from redox reporters, such as Ru(bpy)$_3^{2+}$, which are added externally, so the probes and targets are label-free (Zhou and Rusling, 2001).

Another advantage of using electrochemistry is that the signals can be amplified to achieve extraordinarily high sensitivity, down to attomolar, by increasing the electrode surface area or addition of secondary reporters (Lapierre et al., 2003; Gasparac et al., 2004; Soleymani et al., 2009b).
2.3 Multiplexed sensors

Due to the demand for detection of multiple target biomolecules and genomic sequences, a number of multiplexed sensors that are able to detect various targets simultaneously have been developed.

One of the most important multiplexed devices is the fluorescent microarray first reported in the 1990s, which is able to screen for sets of genes in parallel in a given sample (Fodor et al., 1991; Fodor et al., 1993; Schena et al., 1995; Lockhart et al., 1996). The principle of microarray is similar to that of Northern blots, and other hybridization-based techniques. Nucleic acid probes, usually DNA probes, with various sequences are immobilized on specific locations on the solid surface, such as a glass slide or silicon wafer. Then the fluorescently labeled nucleic acid targets, such as cDNA or RNA, are introduced onto the solid surface. The strands with complementary sequence will be captured by the probe and form the duplex structure according to the Watson-Crick base pairing rules. After washing off the non-specific binding on the surface, the location of probe-target duplexes emits a fluorescent signal. The intensity of the signal is related to the number of fluorescent targets on the surface (Figure 1.4) (Lee and Whitmore, 2002; Simon et al., 2002; Yang and Speed, 2002; Blalock et al., 2003; Guo, 2003; Kerr, 2003; Glonek and Solomon, 2004; Jørstad et al., 2007; Russell et al., 2009).
Figure 1.4. Schematic illustration of cDNA microarray. Multiple target strands could be detected simultaneously with fluorescence labeling. (Figure adapted from Guo, 2003.)

In the scheme shown above, the targets need to be labeled with fluorescent dyes, which is time-consuming and also changes the concentration levels of targets. Several label-free fluorescence microarrays have also been reported (Steemers et al., 2000; Ho et al., 2009).

However, the fluorescence microarray has its own limitations, as described above. The target strands need to be labeled with fluorescent dyes, and this delays the sample-to-answer procedure.
Secondly, the fluorescent dyes are prone to photo bleaching, while some other molecules or materials could show strong fluorescent signals or reflections when exposed under the laser, leading to noisy background signals. Moreover, in multiplexed detection, the targets need to be labeled with different fluorescent dyes with a variety of absorption and emission wavelengths, but the broad absorption and emission window of fluorophores limits the selection of dye-labeling candidates (Medintz et al., 2005).

Instead of using fluorescence, other reporter systems have been tested. One of the alternatives is to detect electrochemical signals from the redox reporters. Several devices have been developed. The basic ones are assemblies of a number of bulk gold electrodes (Figure 1.5).

Figure 1.5. Selected examples of bulk gold electrode assemblies. A number of bulk gold electrodes are assembled on a small silicon wafer or glass slide. Each electrode can be accessed via an outer gold pad. Most of the electrodes are two dimensional without nanostructures. (A) Figure adapted from Slinker et al., 2010. (B) Figure adapted from Laboria et al., 2010. (C) Figure adapted from Levine et al., 2009.
The individual electrodes of the assemblies share the same characteristics. The number of target molecules captured on the surface is proportional to the electrode area. As a two-dimensional platform, the only way to increase the electrode surface is to increase the diameter of the electrode, but electrodes in large diameter often result in high background signals. Therefore, other electrode platforms with large surface and low background signal are required to improve the sensitivity and specificity.

2.4 Nanotechnology and microtechnology in electrochemical sensors

As discussed above, increasing the electrode surface area could increase the maximum number of target molecules captured on the electrode. Construction of electrodes with nano- or microstructures can generate a three-dimensional platform, which increases not only the electrode surface, but also the accessibility of the target molecules and the hybridization efficiency (Soleymani et al., 2009c; Bin et al., 2010). The application of nanotechnology and microtechnology in the fabrication of nanoparticles, nanoelectrodes and microelectrodes could improve the sensitivity significantly (Gasparac et al., 2004).

A variety of sensitive nanoelectrodes and microelectrodes with nanostructures have been constructed from gold, platinum, carbon or other materials (Menon and Martin, 1995; Tu et al., 2003; Koehne et al., 2004; Yang et al., 2006). These electrode assemblies are well suited for fabrication of chip-based electrochemical sensors and for multiple targets detection, which are preferred for establishing automated and high-throughput devices.

In this thesis, chip-based sensors using a bottom-up fabrication strategy to generate multiple electrodes with nanostructures and their applications will be discussed (Fang et al., 2009; Soleymani et al., 2009a; 2009b; 2009c).

3 Summary

Medical diagnosis is an important field for new technological developments. In the long history of medical diagnosis, a large number of techniques have been optimized and are widely used. Among these technologies, genetic analysis is now playing a more and more critical role in disease diagnosis and recovery monitoring. To deal with health problems, such as diarrhea, tuberculosis, malaria, cancer and hospital acquired infection, diagnosis must be done more
efficiently in order to take advantage of all the treatments available. Detection time and cost are the main concerns for development of new test methods.

Nucleic acid sensors using electrochemical readout possess great potential as the solution for providing clinicians with a rapid and affordable diagnostic system. Assembling multiplexed sensors onto a chip-based platform could detect numbers of targets in parallel. In this thesis, we present three different microelectrode platforms, combining electrochemistry, nanotechnology, and microtechnology for direct cancer biomarker detection and rapid bacterial speciation. Multiplexed detection and real time monitoring are achieved on the high throughput chip-based system. The development of these miniaturized, cost-effective tools could be applied for rapid medical and environmental monitoring.
The research presented in the following chapters was performed by Zhichao Fang under supervision of Dr. Shana O. Kelley, unless otherwise indicated. Portions of the texts and figures are adapted from publications co-authored by Zhichao Fang and Dr. Shana O. Kelley (Fang and Kelley, 2009; Fang et al., 2009; Soleymani et al., 2009a; 2009b; 2009c).
Chapter 2
Direct electrocatalytic detection of prostate cancer-related gene fusion using PNA-nanowire sensors

1 Introduction

Due to the growing demand for bioanalytical tools, many different types of bioassays have been developed and are able to detect various biomolecular targets. Bioassays, which convert biological reactions into physicochemical readouts, offer great promise for numerous applications in biochemistry, molecular biology and pharmaceutical sciences (Scheller et al., 2001). Among various biomarkers in disease diagnosis and prognosis, nucleic acids, including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), have been targeted as a critical direction of biosensing development (Baker, 2006). Detection of DNA or RNA by hybridization on a solid support modified with a complementary sequence has been extensively used in various types of bioassays, including those providing optical (Steemers et al., 2000; Erickson et al., 2004; Jin et al., 2006; Fabris et al., 2007; Zeglis et al., 2007; Feng et al., 2008; Willner et al., 2008), gravimetric (Mannelli et al., 2005), piezoelectric (Goodrich et al., 2004; Wu et al., 2007), and electrical or electrochemical readouts (Park et al., 2002; Drummond et al., 2003; Katz and Willner, 2003; Lapierre et al., 2003; Zhang et al., 2003; Gasparac et al., 2004; Hahm and Lieber, 2004; Wang et al., 2004; Lapierre-Devlin et al., 2005; Xiao et al., 2007).

Electrochemical sensors have long been viewed as particularly attractive for bioanalysis for a long time because of their high sensitivity, high selectivity, high speed, low detection limit, low cost, and ease of use (Drummond et al., 2003). While several techniques exist for the quantitative electrochemical detection of DNA (Ozkan et al., 2002; Macanovic et al., 2004; Raymond et al., 2005; Kerman et al., 2006; Liu et al., 2006; Aoki and Tao, 2007; Kerman et al., 2008; Won et al., 2008), most of them mainly rely on multi-step sandwich assays that measure a signal change due to the hybridization of an unmodified target to a probe sequence immobilized on the electrode surface. In addition, most electrochemical sensors can only detect short synthetic DNA strands with detection limits in the nanomolar range. Moreover, some of these devices use a “signal-off” approach that could increase the risk of false positives. The sensitivity of the “signal-on” devices that do exist can be limited by high background signals. These deficiencies are highlighted by the lack of systems that can detect nucleic acids sequences in
heterogeneous samples, where very high sensitivity and the ability to discriminate closely related sequences are required.

Here, we report an electrochemical “signal-on” approach that permits the detection of medically-relevant RNAs in complex biological samples (Figure 2.1). The approach uses peptide nucleic acids (PNAs) plus an electrochemical assay and a nanoscale electrode platform. This system uses two redox reporters for readout, Ru(NH3)₆³⁺, a DNA-binding and cationic electron acceptor, and Fe(CN)₆³⁻, an anionic electron acceptor and electrocatalytic signal amplified reagent. Ru(NH3)₆³⁺ is attracted by the DNA film at the electrode surface through electrostatic interactions with the negatively charged phosphate backbone. During a negative potential sweep, Ru(III) is reduced by the potential and regenerated by the Fe(III) oxidant for multiple turnovers. The multiple redox cycles of Ru(III) amplifies the reduction signal, thus monitors the amount of hybridization at the electrode surface. Previously, we showed that ultrasensitive nucleic acid detection with pM sensitivity could be achieved when an electrocatalytic reporter system was applied in conjunction with DNA probes displayed on nanowire surfaces (Lapierre et al., 2003; Gasparac et al., 2004; Lapierre-Devlin et al., 2005). However, this system proved unable to assay complex mixtures of sequences, and therefore was only useful in a model environment. We therefore sought to improve this method, and to do so, investigated PNA probes, which are uncharged and generate low background signals. Indeed, introducing this material into the assay yields even higher levels of sensitivity, and does so even in the presence of a complex background of non-complementary sequences.
Figure 2.1. (A) Schematic illustration of electrocatalytic detection of nucleic acids using nanowire sensors; (B) Sequences of probes complementary to the TMPRSS2:ERG fusion (P1), wild type TMPRSS2 (P2), or ERG (P3).

2 Experimental section

2.1 Chemicals and materials

Tin(II) chloride (anhydrous, ≥99.99%), trifluoroacetic acid (TFA, ≥98%), silver nitrate (≥99.0%), sodium sulfite (≥98.0%), formaldehyde solution (37 wt. % in H₂O), 1,1’-carbonyldiimidizole, 1,6-hexamethylenediamine, 1,4-dioxane (anhydrous, 99.8%), 3-(2-Pyridyl dithio)propionic acid N-hydroxysuccinimide ester (SPDP), dithiothreitol (DTT), dimethylformamide, m-cresol, triisopropylsilane (TIPS), 6-mercapto-1-hexanol (MCH, 97%), hexaamineruthenium chloride, potassium ferricyanide and potassium ferrocyanide trihydrate were purchased from Sigma-Aldrich Canada Ltd. SPI-Pore polycarbonate “Track-etch” membrane filters (25 mm 0.01 μM) was obtained from SPI supplies (USA). Commercial gold electroless plating solution (Oromerse SO Part B) was purchased from Technic Inc. (USA). DNA oligonucleotides were obtained from
the Centre for Applied Genomics in the Hospital for Sick Children (Toronto, Canada). PNA molecules were purchased from Biosynthesis Inc (USA).

2.2 Preparation of bulk gold electrodes

Bulk gold electrodes were polished using 0.05 \( \mu \)m alumina powder, rinsed in water, sonicated for 5 min, etched by scanning from 0 to 1.8 V at 200 mV/s in 1 M H\(_2\)SO\(_4\), and rinsed with water. The electrodes were dried under nitrogen flow prior to probe immobilization.

2.3 Fabrication of NEEs

Electrodes composed of three-dimensional gold nanowires were fabricated by templated electroless deposition within a polycarbonate membrane and were characterized and optimized according to the methods published previously (Menon and Martin, 1995; Gasparac et al., 2004).

Briefly, the polycarbonate membrane was immersed in a solution containing 26 mM SnCl\(_2\) and 0.07 M TFA in 50/50 methanol/H\(_2\)O for 45 minutes at room temperature, followed by rinsing with methanol. The Sn\(^{2+}\) acted as the “sensitizer” and adhered to the pore walls. The Sn\(^{2+}\)-sensitized membrane was then immersed in an aqueous solution of 29 mM ammoniacal AgNO\(_3\) for 15 minutes at room temperature, followed by rinsing with methanol and water. Then the Ag-coated membrane was immersed in the Au plating bath, which contains 2.5% of the commercial gold electroless plating solution, 0.625 M formaldehyde, and 0.127 M Na\(_2\)SO\(_3\), at 0 \(^\circ\)C for 24 hours. The Au-coated membrane was thoroughly rinsed with water and then immersed in 10% HNO\(_3\) for 12 hours, followed by rinsing in water and air-dried (Figure 2.2).
Figure 2.2. Schematic illustration of electroless Au deposition in the pores of the polycarbonate template membrane. (A) SnCl$_2$, TFA, methanol/H$_2$O, room temperature, 45 min; (B) Ag[(NH$_3$)$_2$]$^+$, room temperature, 15 min; (C) Au$^+$, formaldehyde, Na$_2$SO$_3$, 0 °C, 24 h. (Figure adapted from Menon and Martin, 1995.)

A small piece of the Au-coated membrane was attached to a strip of a sticky copper foil, which acts as the working electrode lead, with the “shiny” side facing up and the rough side facing the adhesive. The upper Au layer was removed by cotton tipped applicators and ethanol, which exposed the disc shaped ends of the Au nanowires. The 2D NEEs were etched in 10 W O$_2$ plasma for 150 seconds using Samco RIE-1C reactive ion etching system. Au nanowires about 200 nm in length and 10 nm in diameter were exposed from the membrane surface after oxygen plasma etching away the polycarbonate material. The 3D NEEs were heated at 150 °C for 30
minutes to produce a water-tight seal between the Au nanowires and the pore walls. Another strip of copper foil was affixed to the gold surface and covered a small part of the membrane, which improved the electrical connection between the copper and the NEEs. Finally, the upper and lower surface was covered by strips of strapping. Prior to placement, a 0.07 cm\(^2\) hole was punched in the upper piece of the tape (Figure 2.3).
The NEEs were etched by scanning from 0 to 1.8 V at 200 mV/s in 0.1 M H$_2$SO$_4$, and rinsed with water. The electrodes were dried under nitrogen flow prior to probe immobilization.
2.4 Preparation and purification of oligonucleotides

The following probe and target sequences were used in experiments employing synthetic oligonucleotides.

DP1 (DNA probe):

\[
\text{SH-}^5' \text{ATA AGG CTT CCT GCC GCG CT } 3'
\]

P1 (PNA probe-1):

\[
\text{NH}_2\text{-Cys-Gly- ATA AGG CTT CCT GCC GCG CT -CONH}_2
\]

P2 (PNA probe-2):

\[
\text{NH}_2\text{-Cys-Gly- TCA ATA TGA CCT GCC GCG CT -CONH}_2
\]

P3 (PNA probe-3):

\[
\text{NH}_2\text{-Cys-Gly- ATA AGG CTT CCT TGA TAT GA -CONH}_2
\]

T1 (TMPRSS2:ERG target):

\[
5' \text{AGC GCG GCA GGA AGC CTT AT } 3'
\]

T2 (TMPRSS2 target):

\[
5' \text{AGC GCG GCA GGT CAT ATT GA } 3'
\]

T3 (ERG target):

\[
5' \text{TCA TAT CAA GGA AGC CTT AT } 3'
\]

T4 (non-complementary target):

\[
5' \text{TTC TTT TTT TTT TTT TTT TT } 3'
\]
TMPRSS2:ERG-F (forward primer for TMPRS2:ERG fusion):

\[ 5' \text{CAG GAG GCG GAG GCG GA} 3' \]

TMPRSS2:ERG-R (reverse primer for TMPRS2:ERG fusion):

\[ 5' \text{GGC ATT GTA GCT GGG GGT GAG} 3' \]

Thiolated DNA probes were prepared and purified following the procedure published previously (Taft et al. 2003). As shown in Figure 2.4, DNA probe DP1 was synthesized using an ABI 394 DNA/RNA synthesizer according to the standard automated solid-phase techniques but was not deprotected and remained on the CPG-resin. The resin was then transferred to a peptide shaker and rinsed with 3 mL of 1,4-dioxane. 200 mg of 1,1-carbonyldiimidazole in 2 mL of 1,4-dioxane was mixed with the resin for 30 min under Argon bubbling, followed by 5 washes with 3 mL of 1,4-dioxane. 200 mg of 1,6-hexamethylenediamine in 2 mL of 1,4-dioxane was mixed with the amine-terminated DNA for 25 min under Ar bubbling to conjugate the amine-terminated linker. After rinsed with 3 mL of 1,4-dioxane 4 times and 20 mL of methanol 3 times, the amine-terminated DNA resin was resuspended in concentrated NH₄OH at 55 °C for 12 hours for deprotection. The solution containing the deprotected DNA was dried and re-dissolved in 200 μL of 0.4 M HEPES buffer (pH 8). Addition of the disulfide linker was carried out by reaction in 4 mg of SPDP and 30% acetonitrile, vortexing for 1 hour. After filtration with a 0.2 μm cellulose acetate microcentrifuge filter, the disulfide DNA was HPLC purified using an Agilent 1100 series HPLC on a Varian Microsorb MV 300-5 C18 column (250 mm * 4.6 mm). 50 mM NH₄Ac and acetonitrile were used as mobile phases. Disulfide DNA was detected by monitoring absorbance at 260 nm and collected. After lyophilized and resuspended in 50 mM sodium phosphate buffer (pH 7), the disulfide group was converted to thiol by reaction with 100 mM DTT for 1 hour. The thiolated DNA was HPLC purified using the same conditions as above and lyophilized. All other unmodified synthetic DNAs were HPLC purified using the same conditions as above.
Figure 2.4. Modification of DNA oligonucleotides for deposition. (a) 1,1’-carbonyldiimidazole, 1,4-dioxane, Ar, 30 min; (b) 1,6-diaminohexane, 1,4-dioxane, Ar, 25 min; (c) conc. NH₃·H₂O, 15 h, 55 °C; (d) SPDP, CH₃CN/0.4 M HEPES (pH 8), 1 h; (e) 100 mM DTT, 50 mM sodium phosphate (pH 7), 1 h.

DNA modification procedure has been tested with different DNA sequences (Taft et al., 2003; Lapierre et al., 2003; Gasparac et al., 2004; Lapierre-Devlin et al., 2005). DNA modified with different terminated groups resulted in different retention time on HPLC (Figure 2.5). On the reverse-phase column, molecules with different polarities show different retention time.
Figure 2.5. HPLC retention time of modified DNA probes. Modified with different functional groups, the DNA molecules have different polarities, resulting in different retention times on the reverse-phase HPLC column.

The PNA probes were synthesized by peptide synthesizer Prelude™ (Protein Technologies, Inc, U.S.A.) using commercially available Knorr resin (LS, 100-200 mesh, 1% DVB). Coupling was achieved using 5 equivalents of Fmoc-protected amino acid or PNA monomer, 7.5 equivalents of HATU and 10 equivalents of N-methylmorpholine in DMF for 3 hours. Deprotection of the Fmoc group was performed using 20% piperidine in DMF for 10 minutes. The PNA probe was cleaved from the resin in the solution containing 85% TFA, 10% m-cresol, 2.5% TIPS, 2.5% H₂O at room temperature for 5 hours, followed by -80 ℃ ether precipitation and air dry overnight (Figure 2.6).
Figure 2.6. Synthesis of PNA oligonucleotides for deposition. (a) HATU, N-methylmorpholine in DMF, 3 hours; (b) 20% piperidine in DMF for 10 minutes; (c) HATU, N-methylmorpholine in DMF, 3 hours; (d) repeat of step (b) and step (c); (e) addition of glycine and cysteine; (f) 85% TFA, 10% m-cresol, 2.5% TIPS, 2.5% H₂O, room temperature, 5 hours.
The thiol group of the cysteine was reduced with 100 mM DTT for 1 hour in 10% acetonitrile. After filtration with a 0.2 μm cellulose acetate microcentrifuge filter, the thiolated PNA probe was HPLC purified using an Agilent 1100 series HPLC on a Varian Microsorb MV 300-5 C18 column (250 mm × 4.6 mm). Water with 0.1% TFA and acetonitrile with 0.1% TFA were used as mobile phases. PNA probe was detected by monitoring absorbance at 260 nm and collected and lyophilized (Figure 2.7).

![HPLC of crude PNA probe cleaved from resin. UV-Vis spectrum of the major peak at approximately 11 min shows the maximum absorbance is at 260 nm (inset).](image)

Before deposition, the PNA probe was resuspended in water and filtered with MicroSpin™ G-25 columns (GE Healthcare, U.K.). The molecular weight of all PNA probes were confirmed by mass-spec (Figure 2.8).
Figure 2.8. Mass spectrum of PP-1. The molecular weight of PP-1 is 5848 g/mol, which matched the peak in mass spectrum, indicating the synthesis of PP-1 probe was successful.
Oligonucleotides were quantitated by measuring absorbance at 260 nm. Extinction coefficients of DNA were calculated from Integrated DNA technologies website (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Extinction coefficients of PNA probes were calculated with the following values: dA = 13 700 M⁻¹ cm⁻¹, dC = 6 600 M⁻¹ cm⁻¹, dG = 11 700 M⁻¹ cm⁻¹, dT = 8 600 M⁻¹ cm⁻¹(http://www.panagene.com).

2.5 Preparation of mRNA and total RNA targets

RNA containing the TMPRSS2:ERG fusion was extracted from the VCaP cell line with the Dynabeads mRNA Direct Kit (Invitrogen, U.S.A.). The mRNA samples from MRC-5 and HeLa cell lines were extracted with the same kit and used as negative controls. Approximately 200 ng mRNA was obtained from 3 million cells, and 10 ng was applied to an electrode for analysis. Total RNA from prostate tumor frozen tissue was provided by Dr. Jeremy A. Squire of the Princess Margaret Hospital (Toronto, Ontario), which was extracted using the Trizol (Invitrogen, U.S.A.) protocol followed by DNase treatment. Approximately 10 μg RNA was obtained from ~50 mg tumor tissue, and 100 ng was applied to an electrode for analysis.

2.6 Modification of bulk gold electrodes and gold nanowires with probe sequences for electrochemical measurements

Single-stranded thiolated DNA probes were immobilized on nanowire electrodes in solution containing 5 μM SH-DNA, 500 nM MCH, 25 mM sodium phosphate (pH 7), 25 mM NaCl and 50 mM MgCl₂ in a dark humidity chamber at room temperature. The deposition time on bulk gold electrode was 30 min, and the deposition time on NEEs was 15 min. To denature the single-stranded thiolated PNA probes before immobilization, they were heated at 55°C for 10 min. They were then applied onto nanowire electrodes in solution containing 10 μM SH-PNA, 1 μM MCH, 25 mM sodium phosphate (pH 7), and 25 mM NaCl in a dark humidity chamber at room temperature overnight. Following deposition, electrodes were rinsed in 25 mM sodium phosphate (pH 7), 25 mM NaCl buffer. MCH is added in the immobilization solution to i) achieve an optimal probe film density, ii) reduce the non-specific adsorption. The adsorption of DNA on the nanowires was confirmed by monitoring the attenuation of the electrochemical signal obtained from a solution of 2 mM ferrocyanide in 25 mM sodium phosphate (pH 7) and 25 mM NaCl solution (data not shown).
2.7 Electrochemical measurements

All cyclic voltammetry measurements were conducted at room temperature with a Bioanalytical Systems Epsilon potentiostat at a scan rate of 100 mV/s. Differential pulse voltammetry (DPV) signals were measured using a potential step of 5 mV, pulse width of 25 ms, pulse period of 100 ms, pulse amplitude of 50 mV; these conditions are equivalent to a scan rate of 50 mV/s. A three-electrode configuration was used consisting of a modified gold working nanowire electrode, a platinum wire auxiliary electrode, and an Ag/AgCl reference electrode.

Electrocatalytic currents were measured when signals reached stable in solutions of 10 μM Ru(NH₃)₆³⁺ and 1 mM Fe(CN)₆³⁻ in 25 mM sodium phosphate (pH 7), and 25 mM NaCl (any deviations from these conditions are described in individual figure captions.) Stable signals were obtained after 2 scans, and thus the second scan was used for quantitation in all experiments. Cathodic charge ($Q$) was quantitated by integrating background-subtracted voltammograms. Signal changes corresponding to hybridization were calculated as follows $\Delta Q (%) = \left(\frac{Q_{\text{final}} - Q_{\text{initial}}}{Q_{\text{initial}}}\right) \times 100\%$. Error bars shown on individual figures correspond to standard error collected from multiple independent trials of each experiment.

2.8 Electrochemical detection of target hybridization

The electrocatalytic current obtained from gold electrodes modified with thiolated probe was measured as described above, the rinsed gold electrodes were then exposed to target sequences, and then hybridization was detected through enhancement of the electrocatalytic signal. Hybridization solutions typically contained target sequences in 25 mM sodium phosphate (pH 7), and 25 mM NaCl. 100 mM MgCl₂ were added during hybridization when DNA probes were used. Electrodes were incubated at 37 °C in a thermostatted humidity chamber in the dark and were washed extensively with buffer before electrochemical analysis. The conditions used for individual experiments varied depending on the size and source of the target nucleic acid; details of different hybridization trials are provided in the figure captions.

2.9 DNA sequencing

The RNA containing the TMPRSS2:ERG fusion was first reversely transcribed to complementary DNA (cDNA) using the High Capacity DNA Reverse Transcription Kit (Applied Biosystems, U.S.A.). The cDNA was further amplified by PCR in a solution containing 1 μM of the primers,
50 U/mL of Taq DNA polymerase (New England Biolabs, U.S.A.) using a thermal cycler, which was set for 40 cycles with the denaturation step at 95 °C for 1 min, the annealing step at 60 °C for 1 min, and the extension step at 72 °C for 1 min. The PCR product was purified by electrophoresis on an Agarose gel. The DNA was extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen Sciences, U.S.A.) and sent to ACGT DNA Technologies Corporation for sequencing.

3 Results and discussion

3.1 Investigation of NEEs fabrication

Fabrication of nanowires was achieved by several methods, one of which is using the pores in microporous filtration membranes as templates. These membranes contain an array of cylindrical pores with uniform diameters, and membranes with various densities and sizes of the pores are commercially available.

The microporous membranes are electronically nonconductive, so the Au is deposited using electroless method. Electroless metal deposition, which uses a chemical reducing agent to reduce the metal ions from solution onto a surface, is mainly applied for coating the metal on the electronically nonconductive surface. The critical requirement of an electroless deposition method is to control the reducing rate of the metal ion to be slow. The reaction used in Au deposition on the microporous membranes is shown below.

\[ 2 \text{Au}(I) + HCHO + 3 \text{OH}^- \rightarrow \text{HCOO}^- + 2 \text{H}_2\text{O} + 2 \text{Au} \]

At room temperature, the Au would simply be reduced in the bulk solution, which turns the solution into dark blue, and the membrane ends up with poor Au coating. Therefore, in order to slow down the reaction, the membrane is immersed in the Au plating bath on ice, and by doing so, the solution stays colorless and the membrane is well coated after 24 hours.

In addition, during the electroless metal deposition, the existence of a catalyst could accelerate the rate of metal ion reduction. The Ag atom is one of the best catalysts in Au reduction. With Ag atoms reduced in the pores, Au could be reduced and filled the cylindrical pores more efficiently.
Oxygen plasma was used to etch the polycarbonate membrane. The length of the Au nanowires could be controlled by the etching time, which was adjusted to expose the Au nanowires at approximately 200 nm in length (Figure 2.9). The 200 nm of Au nanowires are optimal for nucleic acid detection in our system.

![Figure 2.9. Scanning Electron Micrograph (SEM) of gold nanowires. The Au nanowires exposed out of the polycarbonate membrane are approximately 200 nm in length.](image)

Not only can these NEEs be used in DNA detection, they can also serve as a platform for protein detection. (Roberts and Kelley, 2007; Pampalakis and Kelley, 2009).

### 3.2 DNA probe characterization

When the DNA probe is thiolated, it can be immobilized on the bulk gold electrode surface. The DNA modified electrode surface then becomes negatively charged. When the electrode is scanned in an aqueous solution containing Fe(CN)$_6^{4-}$, the negative Fe(CN)$_6^{4-}$ ions are repelled from the electrode surface, so the electron transfer is blocked, leading to the decrease of current (Figure 2.10).
Figure 2.10. Ferrocyanide-blocking effect on DNA modified gold electrodes. When scanned in Fe(CN)$_6^{4-}$, the unmodified gold electrode showed significant redox peaks (blue), while the DNA modified electrode repelled the Fe(CN)$_6^{4-}$ and showed low currents (red), indicating the blocking effect on electron transfer.

### 3.3 Investigation of DNA hybridization on bulk gold electrodes

Our lab has developed a catalytic electroanalytical assay, which provides a sensitive means to detect nucleic acid hybridization with high specificity using electrochemical readout (Lapierre et al., 2003). The method exploits a reaction between Ru(NH$_3$)$_6^{3+}$ (Ruthenium(III)hexaamine), a DNA-binding and redox-active probe molecule, and Fe(CN)$_6^{3-}$ (Ferricyanide). Ru(NH$_3$)$_6^{3+}$ binds to DNA nonspecifically through electrostatic interactions with the phosphate backbone, and therefore, the electrochemical reduction of this species yields a signal that reports on the increase of negatively charged groups at the electrode surface upon hybridization of a target sequence.
The signal is amplified by the Fe(III) oxidant, which permits Ru(III) to be regenerated for multiple redox cycles (Figure 2.11). After hybridization is allowed to occur, the cyclic voltammetry signals should increase because of the larger amount of negative charge on the electrode surface, attracting more Ru(NH$_3$)$_6^{3+}$ cations. To quantitate the DNA hybridization using cyclic voltammetry, the integrated charge of the reduction peak is measured and $\Delta Q$ is calculated based on equation 2.1.

$$\Delta Q = \frac{Q_{\text{final}} - Q_{\text{initial}}}{Q_{\text{initial}}} \times 100\%$$

(Equation 2.1)

Because the electrocatalytic signals are generated by attraction of the Ru(NH$_3$)$_6^{3+}$ with the DNA probe modified electrode surface and independent of the probe sequence, in principle this system could be used in different probe assays and achieve significant signal amplification.

Figure 2.11. (A) Schematic illustration of electrocatalytic reporter systems in nucleic acid detection. (B) Cyclic voltammogram illustrating regular reduction-oxidation signals of DNA modified electrode in 3 mM Ru(NH$_3$)$_6^{3+}$. (C) Cyclic voltammogram illustrating electrocatalytic
signals before (blue line) and after (red line) with 1 μM complementary DNA target under optimal conditions.

Detection limit, or sensitivity, where a signal greater than three times the standard deviation of the control signal is produced, is studied. Under optimal conditions, the detection limit for DNA probe on bulk gold electrodes with electrocatalytic system is 100 nM (Figure 2.12). A positive $\Delta Q$ change can be obtained with 100 nM and 1 μM complementary target, while incubation with 5 μM non-complementary target resulted in negative signal change.

Figure 2.12. Detection limit of DNA probe on bulk gold electrodes. CV was used to quantitate the detection limit for DNA probe on bulk gold electrodes when the complementary target (T1) was introduced in solution. Non-complementary target T4 was used to assess background levels. Hybridization time was 60 minutes. A positive signal change can be obtained down to 100 nM T1. Error bars shown represent standard errors calculated from at least four trials.
3.4 Investigation of the effect of PNA probes on electrocatalytic nucleic acid detection sensitivity

We chose a target sequence for these studies with significant medical relevance, a prostate cancer-related gene fusion, recently discovered to be present in more than 50% of prostate cancer tumors and also thought to have promise as a prognostic factor – patients with this type of gene fusion have greater than 50% chance of cancer recurrence within 5 years after removal surgery. (Nam et al., 2007; Tomlins et al., 2005). While conventional biomarkers for prostate cancer testing, such as the prostate-specific antigen (PSA), have been widely used to diagnose and monitor patients, they lack the ability to provide prognostic information quickly (Stephan et al., 2007). The recently discovered gene fusions are highly significant biomarkers, therefore, as they not only serve as markers of prostate cancer, they can also aid in its classification and could someday guide treatment solutions. Typically, RT-PCR, direct sequencing, or fluorescence in situ hybridization (FISH) is used to detect a gene fusion. These biochemical methods are either time-consuming, or difficult to use with high throughput. We endeavored to develop an assay that would allow fast, ultrasensitive detection of these sequences to further the study of gene fusions as medical indicators.

To generate an assay with high sensitivity and specificity, we looked at a panel of probe sequences and types. We compared two different types of probes: one made of DNA which features a phosphodiester backbone, and one made of peptide nucleic acid (PNA), which has a pseudo-peptide backbone (Egholm et al., 1993; Ratilainen et al., 2000; Brandt and Hoheisel, 2004). Unlike DNA, PNA is a charge-neutral molecule, but just like DNA and RNA, PNA can hybridize with complementary strands, forming right-handed, double-helical complexes according to the Watson-Crick rules. PNA-DNA duplexes are more thermally stable than DNA-DNA duplexes, which improves the efficiency of capture of an oligonucleotide target. The fact that PNA is charge neutral has been used to great benefit in several electrochemical or electronic sensing assays that detect changes in substrate electrostatics to read out the presence of a target sequence (Raymond et al., 2005; Kerman et al., 2006; Liu et al., 2006). We therefore hypothesized that this type of probe could make nanowire-based electrocatalytic nucleic acids detection even more sensitive and robust.

Both DNA (DP1) and PNA (P1) probes featuring the sequence of the prostate cancer related fusion site were made (Figure 2.1B); both contained thiols to facilitate immobilization on gold
nanowires. In addition, a series of synthetic targets were made that permitted not only the sensitivity, but also the specificity of the system to be analyzed. In addition to generating a target 100% complementary to the TMPRSS2:ERG splice site (T1), we also synthesized 50% complementary targets corresponding to the sequences of the unspliced wild-type (wt) genes. The 20-nucleotide targets containing portions of the wt sequences of TMPRSS2 (T2) and ERG (T3) were made to allow the binding of half-complementary targets to be assessed, and in addition, a 20-mer of poly-thymine nucleotides (T4) was made as a completely non-complementary target.

When the DNA probe was immobilized on Au nanowires and challenged with the 100% complementary fusion target (T1) and the 50% complementary wt targets (T2 and T3), significant differences were observed in the magnitudes of electrocatalytic signals observed after hybridization (Figure 2.13A). T1 yielded a much larger signal change than T2 or T3; the half-complementary probe-target pairs yielded distinguishable but small signals compared to the T4 target that has 0% complementarity. Nonetheless, the assay exhibits good specificity and high levels of discrimination against sequences that are not completely complementary.

![Figure 2.13](image.png)

Figure 2.13. Electrocatalytic detection of synthetic DNA targets using (A) DNA-nanowire sensors and (B) PNA-nanowire sensors. Cyclic voltammetry (CV) was used to quantitate charge
at electrodes exposed to different probe/target sequence pairs. (insets) Cyclic voltammograms illustrating electrocatalytic signals before (dotted line) and after (solid line) hybridization with 1 μM complementary DNA target (T1) under optimal conditions (Gasparac et al., 2004). When DNA probes were used, hybridization with T1 was induced in a solution containing 100 nM MgCl₂, 25 mM sodium phosphate (pH 7), and 25 mM NaCl at 37 °C for 30 min. When PNA probes were used, hybridization with T1 was conducted in 25 mM sodium phosphate (pH 7), and 25 mM NaCl at 37 °C for 30 min. Error bars shown represent standard errors calculated from at least four trials.

A number of changes were observed when the DNA probe was replaced by the PNA probe. First, the background signals observed at electrodes with probe-only were significantly decreased (Figure 2.13B). This reflects the fact that the PNA probe is uncharged and therefore unable to facilitate electrocatalysis until a negatively-charged target is captured. This background attenuation has a strong beneficial effect on the performance of the assay, as the suppression of the background signal enhances the signal changes that are observed in the presence of the target. Here, instead of monitoring 50% signal changes, changes over 2000% are observed, which minimizes the possibility of false negative artifact when a base line for sample-to-answer is drawn. The experimental error is similar for trials run with both DNA and PNA probes, but the magnitude of the signal changes corresponding to hybridization increase by more than 40-fold, which indicates that the signal-to-noise ratio improves for this device. In addition, the signals obtained with the half-complementary targets remained low, and the control target T4 did not produce any signal increase. Interestingly, the amount of hybridization observed between the PNA probe and T3 was suppressed relative to that observed with the analogous DNA probe. The fact that T3 gives a lower signal than T2 with PNA probe, even though both targets have the same degree of complementarity with the probe, indicates that another factor must be affecting the results. PNA probes typically form more dense monolayers on Au surface than DNA probes do. Under the conditions used here, PNAs tend to generate monolayers with 3-fold higher densities (Lapierre et al., 2003; Aoki and Tao, 2007); this could then account for the trend observed, as it would be more difficult for the DNA target to penetrate the denser PNA monolayer to reach the buried complementary portion of the probe.
3.5 Detection limit of PNA-nanowire sensors and performance with cellular RNA

Detection limits for electrochemical nucleic acids assays reported in the literature can vary over a wide range, the majority of which are not able to detect targets at concentrations lower than nM. Though there are several examples of methods that exhibit sensitivities to pM and fM concentrations of targets (Zhang et al., 2003; Gasparac et al., 2004; Aoki and Tao, 2007), these are rare, and these levels of sensitivity have not been confirmed in heterogeneous samples with high background levels of non-complementary sequences, i.e. clinical related RNA samples. The electrocatalytic assay used here was originally found to have nM sensitivity when used with bulk gold electrodes and DNA probes (Lapierre et al., 2003). The sensitivity of the assay was significantly improved, with pM concentrations of synthetic targets detectable, when gold nanowires were used as an electrode platform instead again with DNA probes (Gasparac et al., 2004). However, attempts to use this system for detection of oligonucleotides in clinical samples were not successful (data not shown).

In this system, modified only by changing DNA probes to PNA probes, we were able to obtain a detection limit for electrocatalytic detection of a synthetic target probe of 100 fM (Figure 2.14), a significant improvement over previous results (Gasparac et al., 2004). Than we challenged the robustness of this detection limit by introducing a high background of non-complementary RNA pool isolated from the HeLa cell line, a cervical cancer cell line that lacks the fused gene complementary to the probe. In the presence of 0.05 mg/mL of HeLa RNA, the PNA-nanowire sensors were still able to reliably detect 1 pM (7 pg/mL) concentrations of the complementary target. While the detection limit of our system did increase, a very high level of sensitivity was preserved. This level of sensitivity, coupled with outstanding specificity, indicates that this assay is appropriate for biological testing.
3.6 Detection of RNA targets from cell lines and tissue samples

The next step in testing the robustness of the assay was to test whether the gene fusion RNA sequence could be directly detected in RNA samples from cultured human cell lines or not. We acquired VCaP cell line, a prostate cancer derived cell line that contains the gene fusion (Figure 2.15) (Tomlins et al., 2005), and isolated mRNA. We used mRNA extracted from the HeLa
cervical cancer cell line that does not contain the gene fusion as the first negative control. As an additional negative control, we also tested mRNA from the lung fibroblast MRC-5 cell line (Figure 2.16).

Figure 2.15. DNA sequencing result of TMPRSS2:ERG gene fusion (highlighted in blue) in VCaP cell line.
Figure 2.16. Electrocatalytic detection of sequence targets in cellular mRNA. VCaP is the *TMPRSS2:ERG* gene fusion positive cell line; MRC-5 and HeLa are used as negative controls. Hybridization of the mRNA samples with the fusion probe (P1) was monitored, as was the hybridization of VCaP mRNA with the wide type probes P2 and P3. Target solutions containing 10 ng mRNA, 25 mM sodium phosphate (pH 7), and 25 mM NaCl. The preparations of PNA films and hybridization were performed as described in Figure 6. Error bars shown represent standard errors calculated from at least four trials.

Comparison of the electrocatalytic signal changes for the three cell lines clearly showed a differential response for the fusion-positive and fusion-negative cellular mRNA samples. The VCaP cell line, which bears the gene fusion, showed a much larger electrochemical response than either of the fusion-negative cell lines, MRC-5 and HeLa. As little as 10 ng of mRNA at a
concentration of 1 ng/μL produced a positive result, again indicating that this sensing system has a high level of sensitivity even when presented with a complex mixture of RNA sequences.

We confirmed that the response we monitored was truly specific by examining two additional probes (Figure 2.16). A probe complementary to the wild type (WT) TMPRSS2 gene (P2), and one complementary to the WT ERG gene (P3) were monitored along side the fusion probe. Both of the WT probes showed significantly smaller signal changes when challenged with VCaP mRNA, in which the fusion positive RNA is half-complementary to both probes. Just as observed with synthetic targets, the signal of hybridization with P3 was lower than that with P2, further confirming the specificity of the gene-fusion specific signal readout with the corresponding probe (Figure 2.16). These results also demonstrate the importance of providing a panel of probes for the correct identification of the fusion, otherwise, overexpression of WT genes might confound the analysis.

The ultimate application of the method described here is the analysis of patient samples to determine if a tumor tissue sample contains the gene fusion. To explore this application, we tested total RNA samples isolated from prostate tumor tissue. RT-PCR and sequencing of both samples revealed that the TMPRSS2:ERG fusion gene was present in only one of the samples (Figure 2.17). We again challenged our PNA-nanowire sensors to analyze for the presence of the prostate cancer gene fusion, and were able to detect a significant difference in the response of the sensor to the two samples (Figure 2.18, both DPV signals and quantitative results from CV signals were presented). While the fusion negative RNA produced a small signal change corresponding to the levels obtained with the negative controls shown in Figure 8, the fusion positive RNA resulted in a signal increase more than 6 times larger. These experiments, again using a small amount of total RNA (100 ng), indicate that this system provides a means to detect specific RNA sequences in small, clinically relevant samples – a long-standing challenge in the area of electrochemical sensor development. The methodology described here is competitive both in sensitivity and specificity with conventional fluorescence in situ hybridization (FISH) and sequencing approaches to gene fusion identification, while the analysis is much faster and amenable to automation.
Figure 2.17. DNA sequencing result of *TMPRSS2:ERG* gene fusion in patient tumor sample.

Figure 2.18. Electrocatalytic detection of prostate cancer-related gene fusion in total RNA from tumor tissue samples. DPVs illustrating electrocatalytic signals before (dotted line) and after (solid line) hybridization with total RNA from tumor tissue samples. $\Delta Q$ was quantitated from CVs. Target solutions containing 100 ng total RNA, 25 mM sodium phosphate (pH 7), and 25 mM NaCl. Preparation of PNA films and hybridization were performed as described in Figure 6. $\Delta Q$ values listed on plots represent averages collected from at least four trials. Values in parentheses are standard errors.
4 Conclusions

The implementation of low-cost electrochemical sensors in clinical laboratories requires the devices to be both sensitive and selective. Herein, we demonstrate that the use of i) PNA probes, ii) an electrocatalytic reporter system, and iii) a Au-nanowire electrode platform produce such qualities by suppressing the background signals and capturing analyte molecules at low concentrations. This approach is effective even in highly heterogeneous samples, which differentiates it from most other electrochemical sensors. In addition, this assay was able to detect a specific biomarker in tumor RNA, indicating that the selectivity is sufficient for use with clinical samples.

5 Acknowledgement

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Chapter 3
Direct multiplexed detection of cancer biomarkers using a nanostructured microelectrode chip

1 Introduction

Detection of clinical relevant biomarkers using electronic readout has been viewed for a long time as a path to development of integrated, chip-based devices that are cost-effective and sensitivity appropriate for clinical diagnosis (Drummond et al., 2003; Bell, 2004; Weston and Hood, 2004; Ludwig et al., 2005). In principle, devices using electronic readout are sufficiently sensitive to detect small numbers of analyte molecules directly with simple instrumentation. Though tremendous advances in this area and related fields have been developed towards new diagnosis tools, a multiplexed chip that can directly detect biomarkers in clinical samples has yet been reported (Steemers et al., 2000; Cui et al., 2001; Nicewarner-Pena et al., 2001; Park et al., 2002; Katz and Willner, 2003; Bell, 2004; Gasparac et al., 2004; Hahm and Lieber, 2004; Zhang et al., 2004; Munge et al., 2005; Yi et al., 2005; Zheng et al., 2005; Xiao et al., 2007; Vlassiouk et al., 2009). One of the main challenges stems from the difficulty of detecting the target molecules at very low concentration in the presence of high background levels, for instance, when complex biological samples are tested. Moreover, generating multiplexed systems requires high specificity and selectivity.

While nanoparticles, nanowires, and nanotubes used in bioassays have exhibited great improvement in sensitivity of detecting pure targets, they typically fall short of fulfilling the essential selectivity, specificity, and multiplexing in clinical sample analysis. For example, the application of networks of metal nanoparticles interfacing metal microleads displays a broad dynamic range, but with detection limits at 500 fM and unproven performance in heterogeneous samples (Park et al., 2002). Moreover, as field effect transistors, silicon nanowires show applications in detection of various analytes, including DNA and RNA (Cui et al., 2001; Hahm and Lieber, 2004; Gao et al., 2007). However, the application in detection of clinical samples is limited by the low signal-to-background ratio. Nanotubes, especially carbon nanotubes, are widely used as both electrochemical detectors and field effect transistors (Li et al., 2003; Star et al., 2006), but they also lack the sensitivities at concentration levels lower than picomolar, which limits their application without enzymatic amplification.
As described in Chapter 2, we recently reported the direct detection of prostate-related gene fusion in clinical samples using gold nanowire sensors (Fang and Kelley, 2009). This assay exhibits femtomolar sensitivity and is able to detect 100 ng of RNA from prostate tumor tissue without reverse transcription or PCR amplification. However, the difficulty of developing a multiplexing platform based on this assay limits its application in multiple markers profiling and cancer diagnosis. Therefore, a more versatile and robust system is essential to expand the range of availability in clinical diagnostic tools.

2 Experimental section

2.1 Chemicals and materials

Tin(II) chloride (anhydrous, ≥99.99%), trifluoroacetic acid (TFA, ≥98%), silver nitrate (≥99.0%), sodium sulfite (≥98.0%), formaldehyde solution (37 wt. % in H2O), 1,1’-carbonyldiimidizole, 1,6-hexamethylenediamine, 1,4-dioxane (anhydrous, 99.8%), 3-(2-Pyridyl dithio)propionic acid N-hydroxysuccinimide ester (SPDP), dithiothreitol (DTT), dimethylformamide, m-cresol, triisopropylsilane (TIPS), 6-mercapto-1-hexanol (MCH, 97%), hexaamineruthenium chloride, potassium ferricyanide and potassium ferrocyanide trihydrate were purchased from Sigma-Aldrich Canada Ltd. DNA oligonucleotides were obtained from the Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Canada).

2.2 Chip fabrication

Chips were fabricated at the Canadian Photonics Fabrication Center. Three-inch silicon wafers were passivated using a thick layer of thermally grown silicon dioxide. A 350 nm gold layer was deposited on the chip using electron-beam-assisted gold evaporation. The gold film was patterned using standard photolithography and lift-off process. A 500 nm layer of insulating silicon dioxide was deposited using chemical vapor deposition; 500 nm apertures were imprinted on the electrodes using standard photolithography, and 2 mm × 2 mm bond pads were exposed using standard photolithography (Figure 3.1).
2.3 Fabrication of nanostructured microelectrodes

Chips were cleaned by rinsing in acetone, IPA, and DI water for 30 s and dried with a flow of nitrogen. All electrodeposition was performed at room temperature using Bioanalytical Systems Epsilon potentiostat with a three-electrode system featuring a platinum wire auxiliary electrode and a Ag/AgCl reference electrode; 500 nm apertures on the fabricated electrodes were used as the working electrode and were contacted using the exposed bond pads. Palladium NMEs were fabricated in a palladium bath containing 5 mM solution of $\text{H}_2\text{PdCl}_4$ and 0.5 M $\text{HClO}_4$ at -250 mV for 10 s using DC potential amperometry.

2.4 Preparation and purification of oligonucleotides

The following probe and target sequences were used in experiments.

Seq. DP1 (DNA probe – type III fusion):

$$\text{SH}^{-5'} \text{ATA AGG CTT CCT GCC GCG CT}^{3'}$$
Seq. P1 (PNA probe – type III fusion):

\[
\text{NH}_2\text{-Cys-Gly- ATA AGG CTT CCT GCC GCG CT -CONH}_2
\]

Seq. P2 (PNA probe – type I fusion):

\[
\text{NH}_2\text{-Cys-Gly- CTG GAA TAA CCT GCC GCG CT -CONH}_2
\]

Seq. P3 (PNA probe – type VI fusion):

\[
\text{NH}_2\text{-Cys-Gly- ATA AGG CTT CTG AGT TCA AA -CONH}_2
\]

Seq. T1 (DNA target – type III fusion):

\[
5' \text{AGC GCG GCA GGA AGC CTT AT} 3'
\]

Seq. T2 (DNA target – WT \text{TMPRSS2)/}:

\[
5' \text{AGC GCG GCA GGT CAT ATT GA} 3'
\]

Seq. T3 (DNA target – WT \text{ERG)/}:

\[
5' \text{TCA TAT CAA GGA AGC CTT AT} 3'
\]

Seq. T4 (DNA target – non-complementary):

\[
5' \text{TTT TTT TTT TTT TTT TT} 3'
\]

DNA and PNA probes and targets were prepared and purified as described in Chapter 2 - 2.4.

### 2.5 Preparation of mRNA and total RNA targets

mRNA containing the \text{TMPRSS2:ERG} fusion was extracted from the VCaP cell line with the Dynabeads mRNA Direct Kit (Invitrogen, U.S.A.). Two typical prostate cancer tissue samples were obtained from radical prostatectomies collected from the Cooperative Human Tissue Network. The tissue was stored at -85 °C until tumor-rich tissue was selected for mRNA extraction. The concentrations of mRNA targets were measured by NanoDrop ND-1000 of thermo Fisher Scientific (U.S.A.). All of the fusion sequences were confirmed by RT-PCR and direct sequencing.
2.6 Modification of NMEs with PNA probes

A solution containing 500 nM thiolated single-stranded PNA, 25 mM sodium phosphate (pH 7), and 25 mM sodium chloride was heated at 50 °C for 10 min. A suitable amount of 10 mM MCH was then added to make the final MCH concentration of 100 nM; 0.5 – 10 μL (depending on the degree of multiplexing) of this mixture was deposited on the NMEs in a dark humidity chamber overnight at 4 °C. The NMEs were rinsed in 25 mM sodium phosphate (pH 7) and 25 mM NaCl buffer before measurements.

2.7 Electrochemical measurements

Electrocatalytic currents were measured when signals stabilized in solutions of 10 μM Ru(NH₃)₆³⁺ and 4 mM Fe(CN)₆³⁻ in 25 mM sodium phosphate (pH 7), and 25 mM NaCl (any deviations from these conditions are described in individual figure captions.) Stable signals were obtained after 2 scans, and thus the second scan was used for quantitation in all experiments.

Differential pulse voltammetry (DPV) signals were measured with a Bioanalytical Systems Epsilon potentiostat using a potential step of 5 mV, pulse width of 50 ms, pulse period of 100 ms, pulse amplitude of 50 mV; these conditions are equivalent to a scan rate of 50 mV/s. A three-electrode configuration was used consisting of a modified gold working nanowire electrode, a platinum wire auxiliary electrode, and an Ag/AgCl reference electrode. Cyclic voltammetry measurements were conducted at room temperature at a scan rate of 100 mV/s. Limiting reductive current (I) was quantified by subtracting the background at 0 mV from the cathodic current at -300 mV in a cyclic voltammetry signal. Signal changes corresponding to hybridization were calculated as follows $\Delta I (%) = [(I_{ds} - I_{ss}) / I_{ss}] \times 100\%$ (ss = before hybridization, ds = after hybridization).

2.8 Hybridization protocol

Hybridization solutions typically contained target sequences in 25 mM sodium phosphate (pH 7), and 25 mM NaCl. 100 mM MgCl₂ were added during hybridization when DNA probes were used. Electrodes were incubated at 37 °C in a dark humidity chamber in dark for 60 min and were washed extensively with buffer before electrochemical analysis.
2.9 Kinetic Measurements of DNA hybridization at NME

PNA (seq. P1)-modified NMEs were prepared as described above. Rinsed NMEs were immersed in a solution containing 10 μM Ru(NH$_3$)$_6^{3+}$, 4 mM Fe(CN)$_6^{3-}$, 100 fM DNA target in 25 mM sodium phosphate (pH 7), and 25 mM NaCl buffer. The electrocatalytic CV signals were obtained as described above. All measurements were performed at 37 °C.

3 Results and discussion

3.1 Comparison of electroplating and electroless deposition

Generation of nanowires in NEEs fabrication was achieved by electroless deposition of Au in the pores of microporous filtration membranes, while electroplating was used in fabrication of the NMEs. Both electroplating and electroless deposition are widely used in fabrication of micro- and nanoelectrodes. A comparison of both methods is listed below (Table 3.1).
### Table 3.1. Comparison between electroplating and electroless deposition

<table>
<thead>
<tr>
<th></th>
<th>Electroplating</th>
<th>Electroless deposition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Equation</strong></td>
<td>$\text{M}^{2+}<em>{(aq)} + e \rightarrow \text{M}</em>{(s)}$</td>
<td>$\text{M}^{2+}<em>{(aq)} + \text{Red}</em>{(aq)} \rightarrow \text{M}_{(s)} + \text{Oxy}$</td>
</tr>
<tr>
<td><strong>Reaction</strong></td>
<td>Non-spontaneous, need to apply external potential</td>
<td>Spontaneous, starts immediately after mixing the metal cation and reducing agent</td>
</tr>
<tr>
<td><strong>Reaction rate</strong></td>
<td>Can be controlled by chemical kinetics and applied potential</td>
<td>Can only be controlled by chemical kinetics</td>
</tr>
<tr>
<td><strong>Set up</strong></td>
<td>Electrolytic cell (including two or three electrodes, electrolyte solution, and external source of current)</td>
<td>Deposition solution and deposition surface, no external source of electrical current</td>
</tr>
<tr>
<td><strong>Deposition solution</strong></td>
<td>Metal cation</td>
<td>Metal cation and reducing agent</td>
</tr>
<tr>
<td><strong>Plating surface</strong></td>
<td>Must be conductive</td>
<td>Can be non-conductive</td>
</tr>
<tr>
<td><strong>Plating position</strong></td>
<td>Can be directed.</td>
<td>Whole surface.</td>
</tr>
<tr>
<td><strong>Application</strong></td>
<td>Fabrication of NMEs</td>
<td>Fabrication of NEEs</td>
</tr>
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### 3.2 Fabrication of NMEs

Several requirements have to be satisfied in order to generate a nanomaterial-based platform for ultrasensitive bioanalysis, including i) highly robust and straightforward to fabricate, ii) multiplexed and scalable, and iii) sensitive and specific when presented with heterogeneous biological samples. Requirements i) and ii) need a mean of achieving reproducible placement of each individual sensing element using a scalable protocol. Requirement iii) needs to incorporate
nanoscale features into the sensing array. Prior work completed by other members in our lab has illustrated that nanostructures have great improvements for biomolecular sensing, which is consistent with the hypothesis that the capture of biomolecules in solution can be rendered inefficient when the probe monolayers are immobilized on bulk surface (Gasparac et al., 2004; Lapierre-Devlin et al., 2005). However, the fabrication of nanostructure assays could be labor-intensive and prone to low reproducibility. Electron-beam lithography indeed provides fine control over nanoscale features and their placement, but it is a serial technique that is not presently suitable to be applied for low-cost and mass-produce chip production.

In order to develop a cost-effective method to fabricate nanostructured microelectrodes, we used conventional photolithography to position and address our electrodes and then apply electrodeposition for generating the nanostructuring of these microelectrodes. Figure 3.4 shows schematic illustration of the 8-fold multiplexed chip. A 350 nm thick gold layer was patterned on a silicon chip wafer to create 8 * 5 μm wide Au wires attached to large metal pads that could serve as external contacts. A layer of SiO₂ was deposited to cover the Au wires as a passivating layer. An aperture of 500 nm in diameter was created at the end of each Au wire, which was used as individual templates for controlled, local growth of nanostructures. Palladium (Pd) microelectrodes were electrodeposited in the patterned apertures (Figure 3.4). The size of the nanostructures could be controlled by varying the deposition time. The diameter of the Pd structures was confined to the ultramicroelectrode regime (< 10 μm). Under conditions for rapid metal deposition, the surfaces of the microelectrodes showed a high level of nanostructuring, with features sized from approximately 20 nm. These nanostructures are proposed to increase the probe surface coverage and promote the hybridization efficiency (Soleymani et al., 2009a; Bin et al., 2010). This type of metal structures displayed ideal microelectrode behavior, exhibiting low capacitive currents and high steady-state plateau current (Figure 3.2)
Figure 3.2. Time dependence of Pd NME electrodeposition. The diameter and height of the NMEs as visualized with scanning electron microscopy (SEM) are controlled by manipulating deposition time. In addition, cyclic voltammograms (CVs) for 3 mM Ru(NH₃)₆³⁺ demonstrate that larger electrode area is obtained with longer electrodeposition time. Approximate apparent microelectrode diameters were calculated from limiting current values.

3.3 Programmable nanostructuring fabrication

The nanostructure size and topography can be programmed using the axes of electroplating time, potential, reagent concentration and supporting electrolytes. When low potentials were used, microelectrodes produced were very smooth; with more negative potentials, microelectrodes with extensive nanotexturing were generated (Figure 3.3A). Applying more negative potentials accelerates the electrodeposition kinetics, resulting in rougher surfaces where growth proceeds outwards faster than internal voids can be filled in. The supporting electrolyte used in electroplating bath also played an important role in microelectrode nanostructuring (Figure 3.3B-D). Electrodeposition in the presence of HCl produced structures that were dense and nanorough.
on the scale of 100-300 nm, while when HClO₄ was used, finer structuring and nanorough on the scale of 20-50 nm were generated. In addition, the electrodeposition rate in HClO₄ was much faster than that in HCl, as the plating time to produce an NME of 8 μm in diameter in HClO₄ (40 sec) was much shorter than that in HCl (500 sec) (Figure 3.3B, C).

Figure 3.3. Programmable NMEs. (A) More negative plating potentials creates more extensive nanostructures. Structures are fabricated (from left) at 0 mV, -100 mV, and -250 mV in HCl for 150 sec. (B) Effect of deposition time on the structure size. The same aperture is imaged (from left) after 20 s, 125 s, and 500 s of plating at -100 mV in HCl. (C) Effect of deposition time and supporting electrolyte on plating rate. Structures are plated for (from left) 5 s, 10 s, and 40 s at -100 mV in HClO₄ as supporting electrolyte. (D) Effect of palladium ion/ HCl ratio on NME morphology. Structures are fabricated (from left) in 5 mM H₂PdCl₄ + 60 mM HCl, 5 mM
H₂PdCl₄ + 30 mM HCl, and 5 mM H₂PdCl₄ + 15 mM HCl, at -100 mV for 150 s. (research performed by Leyla Soleymani)

HCl is an inhibitory electrolyte that slows the growth of the NMEs by suppressing the ionization of the palladium(II) salt. However, when HClO₄ is used as an electrolyte, growth proceeds without inhibition and the fast electrodeposition that is therefore observed yields more fractal structures as the growth of tree-like structures occurs efficiently.

NMEs in Figure 3.3D were electroplated with 5 mM palladium(II) with different concentrations of HCl. When the electroplating bath was prepared, palladium(II) was introduced in the form of H₂PdCl₄. As shown below, H₂PdCl₄ forms a number of complexes in aqueous solution. The stepwise stability constants (Kₙ) of each equilibrium and the overall stability constants (βₙ) had been reported in the previous literature (Elding, 1972).

\[
\begin{align*}
PdCl_4^{2-} + H_2O & \rightleftharpoons Pd(H_2O)Cl_3^- + Cl^- & \log K_4 = 1.37 & \log \beta_4 = \log (K_1K_2K_3K_4) = 11.54 \\
Pd(H_2O)Cl_3^- + H_2O & \rightleftharpoons Pd(H_2O)_2Cl_2 + Cl^- & \log K_3 = 2.41 & \log \beta_3 = \log (K_1K_2K_3) = 10.17 \\
Pd(H_2O)_2Cl_2 + H_2O & \rightleftharpoons Pd(H_2O)_3Cl^+ + Cl^- & \log K_2 = 3.29 & \log \beta_2 = \log (K_1K_2) = 7.76 \\
Pd(H_2O)_3Cl^+ + H_2O & \rightleftharpoons Pd(H_2O)_4^{2+} + Cl^- & \log K_1 = 4.47 & \log \beta_1 = \log (K_1) = 4.47
\end{align*}
\]

The distribution of \([Pd(H_2O)_{4-n}Cl_n]^{2-n}\) is shown in Figure 3.4, calculated using the Equations 3.1-3.5.
Figure 3.4. Distribution of Pd(II) complexes as a function of the concentration of Cl\(^-\). The percentage of PdCl\(_4^{2-}\) increases with the increase of Cl\(^-\) concentration.

\[
x_0 = \frac{\left[ \text{Pd}(\text{H}_2\text{O})_4^{2+} \right]}{c(\text{Pd})} = \frac{1}{1 + [\text{Cl}^-] \beta_1 + [\text{Cl}^-]^2 \beta_2 + [\text{Cl}^-]^3 \beta_3 + [\text{Cl}^-]^4 \beta_4}
\]  \hspace{1cm} \text{(Equation 3.1)}

\[
x_1 = \frac{\left[ \text{Pd}(\text{H}_2\text{O})_3\text{Cl}^+ \right]}{c(\text{Pd})} = \frac{[\text{Cl}^-] \beta_1}{1 + [\text{Cl}^-] \beta_1 + [\text{Cl}^-]^2 \beta_2 + [\text{Cl}^-]^3 \beta_3 + [\text{Cl}^-]^4 \beta_4}
\]  \hspace{1cm} \text{(Equation 3.2)}

\[
x_2 = \frac{\left[ \text{Pd}(\text{H}_2\text{O})_2\text{Cl}_2^- \right]}{c(\text{Pd})} = \frac{[\text{Cl}^-]^2 \beta_2}{1 + [\text{Cl}^-] \beta_1 + [\text{Cl}^-]^2 \beta_2 + [\text{Cl}^-]^3 \beta_3 + [\text{Cl}^-]^4 \beta_4}
\]  \hspace{1cm} \text{(Equation 3.3)}

\[
x_3 = \frac{\left[ \text{Pd}(\text{H}_2\text{O})\text{Cl}_3^- \right]}{c(\text{Pd})} = \frac{[\text{Cl}^-]^3 \beta_3}{1 + [\text{Cl}^-] \beta_1 + [\text{Cl}^-]^2 \beta_2 + [\text{Cl}^-]^3 \beta_3 + [\text{Cl}^-]^4 \beta_4}
\]  \hspace{1cm} \text{(Equation 3.4)}

\[
x_4 = \frac{\left[ \text{PdCl}_4^{2-} \right]}{c(\text{Pd})} = \frac{[\text{Cl}^-]^4 \beta_4}{1 + [\text{Cl}^-] \beta_1 + [\text{Cl}^-]^2 \beta_2 + [\text{Cl}^-]^3 \beta_3 + [\text{Cl}^-]^4 \beta_4}
\]  \hspace{1cm} \text{(Equation 3.5)}
The reduction potential of $\text{PdCl}_4^{2-}$ is determined by the concentration of $\text{PdCl}_4^{2-}$ and $\text{Cl}^-$, according to Nerst’s equation (Equation 3.6, Figure 3.5). 

$$E_{\text{red}} = E^{\theta} - \frac{0.0591}{2} \log \frac{[\text{Cl}^-]^4}{[\text{PdCl}_4^{2-}]} \quad (\text{Equation 3.6})$$

$$E^{\theta}(\text{Pd/PdCl}_4^{2-}) = 0.621 \text{V}$$

As shown in Figure 3.5, with a higher concentration of $\text{Cl}^-$, the reduction potential decreases (blue curve), while the plating potential was kept constant at -100 mV (red line), so the overpotential ($\Delta E$), which means the difference between plating potential and the reduction potential, decreased. Therefore, the electroplating kinetics decelerated, producing smaller NMEs (Figure 3.5D).

![Figure 3.5. Reduction potential of $\text{PdCl}_4^{2-}$ (blue curve) as a function of the concentration of $\text{Cl}^-$. HCl acts as an inhibitory electrolyte.](image)

### 3.4 Detection limit of PNA probe device

In order to test the nanostructured microelectrodes (NMEs) function as nucleic acid bioassays, we modified them with thiolated peptide nucleic acid (PNA) probes. The application of PNA as a
probe molecule has been shown to improve the detection limit of bioassays (Liu et al., 2006; Kerman et al., 2008). Moreover, it is particularly advantageous in electrochemical assays in the way that it lowers the background currents since it is charge neutral. To monitor nucleic acid hybridization with an electrical signal, we employed an electrocatalytic reporter system, which was developed by our laboratory (Lapierre et al., 2003). It relies on the accumulation of Ru(NH₃)₆³⁺ at the electrode surfaces when modified by polyanionic species such as nucleic acids and the catalysis of reduction of Ru(III) via the inclusion of Fe(CN)₆³⁻, which regenerates Ru(III) and leads to multiple reductions per metal center. When the NMEs surfaces were modified with uncharged PNA, this electrocatalytic reporter system did not set up and the background signals were low. Until the PNA-modified NMEs were challenged with a complementary nucleic acid molecules, detectable electrocatalytic signal changes could be clearly obtained down to 10 aM of DNA target (Figure 3.6, research performed and data analyzed by Leyla Soleymani). Incubation with completely noncomplementary sequences resulted in negligible system noise.

Figure 3.6. Signal to background ratio of a PNA probe device (left) and a DNA probe device (right) at different concentrations of complementary target. The system noise associated with hybridization with noncomplementary target is shown for reference. The devices contained 0.1 µM MCH acting as a spacer. (Research performed by Leyla Soleymani.)
3.5 Detection of prostate cancer-related gene fusion using PNA-modified NMEs.

A group of gene fusions specific to prostate cancer was selected as the target biomarkers for analysis on this platform. These gene fusions, resulting from a chromosomal translocation that joins the \(TMPRSS2\) and \(ERG\) genes (\(TMPRSS2:ERG\)), were recently discovered, which appear in more than 50% of prostate tumors (Tomlins et al., 2005; Kumar-Sinha et al., 2008). In addition, there are approximately 20 sequence types that feature different fusion sites and the exact type of fusion in a tumor appears to correlate with its aggressiveness and metastatic potential, which indicates the presence of these gene fusions is not only a promising diagnostic factor but also a valuable prognostic factor (Wang et al., 2006b).

The \(TMPRSS2:ERG\) gene fusion sequences need to be discriminated from the wild-type sequences. We investigated the specificity of our assay with NME sensors modified with a probe complementary to the splice site of the most common type III fusion challenged with i) the \(TMPRSS2:ERG\) fusion target (seq. T1), ii) the wild-type \(TMPRSS2\) gene sequence (seq. T2), and iii) the wild-type \(ERG\) gene sequence (seq. T3) (Figure 3.7). A completely noncomplementary control sequence was also assayed (seq.T4). With hybridization time of 60 min, large signal increases were observed with the fully complementary fusion gene sequence target, while the \(TMPRSS2\) target displayed a much lower signal change. The \(ERG\) target resulted in an even lower signal change, and that of the noncomplementary target was negligible. The \(TMPRSS2\) target binds to the portion of the probe located at the top-half end of the sequence not attached to the electrode, while the \(ERG\) target binds to the portion of the probe located at the bottom-half end tethered to the electrode surface. The different signal levels obtained may indicate that the most accessible side of the probe is able to better capture incoming target molecules, while hybridization with the more buried part of the probe is inefficient since the target molecule has to penetrate deep into the probe monolayer. Furthermore, the fact that the noncomplementary portion of T2 has higher homology to the probe may also contribute to the higher background level. A recent study of our laboratory using the similar platform displayed the ability to discriminate sequences that differ by only a single nucleotide, verifying that the selectivity of these sensors is high (Yang et al., 2009).
Figure 3.7. Differentiation of related sequences: a fully complementary target (T1) corresponding to the type III gene fusion, half-complementary targets (T2 and T3) corresponding to the wild-type *TMPRSS2* and *ERG* genes, respectively, and non-complementary target T4; 100 fM of DNA targets in 25 mM sodium phosphate (pH 7) and 25 mM NaCl was incubated at the PNA-modified NMEs at 37 °C for 60 min. Error bars shown represent standard errors calculated from at least four trials.

3.6 Measurement of real time hybridization kinetics.

In order to determine the hybridization of different targets needed the full 60 min time period originally tested for accurate readout, the electrocatalytic signals of the same NME were monitored in real time (Figure 3.8). The rise of the signals for all types of targets is very fast, and significant current changes were observed within 2 min. However, over the total 60 min time
period, the signals for the half-complementary and noncomplementary sequences fall noticeably, with 20 - 50% of the 2 min signal vanishing by 60 min, while the signals for the fully complementary sequence continued to increase until reaching a plateau after 20 min. It appears that, for sequences that are not fully complementary, some non-specific adsorption occurs in the first few minutes of exposure of the NME sensor to the target solution, but these complexes are not stable enough to remain immobilized on the electrode surface. Therefore, while noncomplementary sequences can be discriminated from complementary sequences within short hybridization time periods, longer times increase the differential signal changes and thus improve the degree of specificity.

Figure 3.8. Kinetics of DNA hybridization at NMEs. Ru(III)/Fe(III) electrocatalysis was used to monitor signal changes when PNA-modified NMEs were incubated with 100 fM of DNA targets with different complementarities. The hybridization signal for fully complementary targets continued to increase until reaching a plateau after 20 min, while other targets with partial or no complementarity showed large initial signals with attenuation of the signal over time. Hybridization performed under the same conditions as those described in Figure 3.7. Error bars
shown represent standard errors calculated from at least four trials. Data fits are shown to illustrate general behavior of the time dependence and do not represent a specific kinetic model.

Two differently nanostructured microelectrodes were also tested. The more nanostructured sensor exhibited a very fast response, rising to 80% of its maximal signal within 5 minutes, while the coarser and denser structure took significantly longer to generate the same signal change, indicating that hybridization is slower when probes are displayed on this structure (Figure 3.9). These results illustrated that immobilized biomolecules with 5-10 nm length scales are more accessible when the monolayer was formed on structures that are finely featured on a similar length scale.
Figure 3.9. Hybridization kinetics observed for two different types of NMEs. NMEs with moderate (red) and fine (blue) nanostructuring were studied by measuring the time evolution of $\Delta I$ while immersing the PNA-modified NMEs in solutions containing 100 fM synthetic target and the Ru(III)/Fe(III) reporter system. Error bars shown represent standard errors calculated from at least four trials.
3.7 Multiplexed detection of a panel of prostate cancer-related gene fusions.

The performance of these NMEs as nucleic acid sensors demonstrated that the patterned structures were indeed sensitive and specific when used under appropriate hybridization conditions. We then sought to prove that multiplexed chip-based NMEs could be used in cancer biomarker detection in heterogeneous biological samples. To test this capability, extracts from cell lines and from tumor samples of prostate cancer patients were assessed to determine whether the sensitivity and specificity of the system was robust enough for clinical testing. We first tested mRNA isolated from two prostate cancer cell lines: VCaP and DU145. The former cell line is type III fusion positive, and the latter one is fusion negative (Tomlins et al., 2005). mRNA was chosen as the target biomolecule over DNA because i) mRNA molecules are mostly single-stranded compared to DNA molecules that are double-stranded and form compact structures with histones; ii) the copy number of mRNA is much larger than that of DNA; iii) the expression level of mRNA is more correlated to the functional characteristics of the gene. As shown in Figure 3.10, no appreciable signal changes observed when 10 ng of mRNA from the DU145 cell line was incubated with an NME modified with a probe complementary to the type III fusion (seq. P1), while significant signal increases were obtained in the presence of 10 ng of mRNA from the VCaP cell line, which does contain the type III fusion. Moreover, NMEs were modified with a probe complementary to a different fusion (seq. P2) did not yield a large signal with type III fusion positive mRNA sample. Therefore, the detection of the gene fusion is highly specific and the results are significant, as the efficiency in the use of 10 ng of the mRNA sample and the total time required for analysis (less than 90 min) are advantageous over other detection methods such as fluorescence in situ hybridization (FISH) and DNA sequencing.
Figure 3.10. Detection of a prostate-cancer-associated gene fusion in VCaP RNA. 10 ng RNA from VCaP (fusion positive) or DU145 (fusion negative) was incubated with PNA probe-modified NMEs for 60 min at 37 °C. The VCaP RNA, when matched with an NME bearing a probe complementary to the type III gene fusion (Seq. P1), produced a large change in signal (center plot), while if a type I probe (Seq. P2) was used instead, no change in signal was observed (left inset). The DU145 RNA also did not produce an appreciable change in the electrochemical signal when incubated with a type III probe (right inset). These controls verify that the hybridization observed with VCaP RNA and the type III probe is specific.

The ultimate application of the NMEs chip is the direct, multiplexed analysis of a panel of cancer biomarkers in clinical relevant samples. Herein, we tested the mRNA samples extracted from cell lines and clinical tumor samples for a series of \textit{TMPRSS2:ERG} gene fusions (Figure 3.10). We obtained a group of samples that contain the three most common types of prostate cancer-
related *TMPRSS2:ERG* fusion: type I, type III, and type VI. Different clinical outcomes are associated with different types of gene fusion. Type III fusions are the most common but correlate with low cancer recurrence rates, while type I and type VI fusions are correlated with aggressive cancers with high levels of recurrence (Wang *et al.*, 2006b). Therefore, it is of great interest to develop an assay that is able to screen different fusions in tumors and to permit their presence or absence to be assessed quickly and straightforwardly, which would be valuable in their further study and validation as diagnostic biomarkers.

PNA probes complementary to each of the three fusions were deposited on the electrodes on the chips respectively, and five different mRNA samples were profiled for the presence of different gene fusions in a multiplexed format (Figure 3.11). Three cell line samples were tested: DU145 (fusion negative), VCaP (type III positive) (Tomlins *et al.*, 2005), and NCI-H660 (type III and VI positive) (Mertz *et al.*, 2007). Furthermore, two tumor samples (tissues collected by radical prostatectomies) were tested: one was positive for type I fusion and the other was positive for type III fusion, as confirmed by conventional sequencing. In all of the cases, the experiments took less than 1.5 hours and required only 10 ng of mRNA. By analyzing the electrochemical signals collected at NMEs modified with different probes, we ascertained the identity of fusion genes present in each sample. In the patient sample containing the type I fusion (as verified by sequencing), the current values observed at each probe-modified NME displayed in the following order: I $>>$ III $>$ VI. In the patient sample containing the type III fusion, the electrochemical signals again illustrate the correct identity of the fusion with probe III $>>$ I $>$ VI. All of the results with patient samples and cell line samples indicate that NMEs chips are able to profile these important biomarkers in heterogeneous samples and to distinguish biomarker profiles associated with different clinical outcomes.
Figure 3.11. Multiplexed profiling of prostate cancer-related gene fusions in cell lines and clinical samples. Three cell lines and two patient samples were analyzed for three different types of prostate-cancer-related gene fusions: type I, type III, and type VI. 10 ng of mRNA isolated from three cell lines were tested: VCaP (type III positive), NCI-H660 (type III and type VI positive), and DU145 (fusion negative). In addition, two tumor samples were tested, one that was positive for the type I fusion, and one that was positive for the type III fusion. Probes corresponding to the splice sites of each fusion sequence (Seq. P1, P2, and P3) were immobilized on separate NMEs on a chip, along with a control (C), and electrocatalytic signals were collected after 60 min hybridization with RNA samples. The RNA solutions had a concentration of 1 ng/μL Sequences that were also observed after PCR and direct sequencing are highlighted as black bars. Error bars shown represent standard errors calculated from at least four trials. The inset depicts an image of the multiplexed chip used for the analysis, which had a type I, type III, type VI, and control (C) probe immobilized on separate NMEs.
4 Conclusions

The NMEs chip format is not only specific, sensitive, and robust, but also practical and scalable. The fabrication method, which is reproducible and easy to conduct, is amenable to the production of probe-modified chips using the same photolithographic technologies in widespread use of consumer electronics microchip fabrication, and only simple, low-cost instrumentation is required for data readout. Other accessories, such as microfluidics, are not necessary for automated analysis, as hybridization can be performed and read out in a single reaction vessel. This system illustrates an attractive alternative to PCR-based assays that are sensitive but difficult to automate in a clinical setting.

In summary, the multiplexed microelectrode platform described here is the first to read directly a panel of cancer biomarkers in clinically relevant samples using electrochemical signals. The array enables these measurements with microelectrodes that possess controllable and versatile nanostructuring essential for sensitivity. This system combines nanotextured electrodes with rapid catalytic readout to achieve a long-standing goal: the multiplexed analysis of cancer biomarkers using a low-cost and practical platform.

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Chapter 4
Rapid and sensitive bacterial speciation in clinically relevant samples using an integrated circuit

1 Introduction

Bacterial infections have caused thousands of deaths and millions of dollars lost around the world annually. It is estimated that 5-10% of all hospital patients acquire some types of nosocomial infection (Tortora \textit{et al.}, 2001). At present, the bacteria causing infections are classified or identified using methods listed in Table 1.3. Most of the methods are not rapid and sensitive enough to directly analyze the DNA or RNA sequence of the bacteria, so culturing is a required step, which is too slow for halting transmission.

Another problem is the reliability of the test results. Due to the rapid gene mutation of the bacterial strains and gene transfer between different types of bacteria, the test results of some conventional methods, such as phenotypic tests of antibiotic resistance, can be unreliable after 24-48 hours (Wax \textit{et al.}, 2008). Speciation of the bacteria based on their genome sequences is a more reliable approach. PCR and DNA sequencing methods are commonly used in bacterial nucleic acid analysis. Specific gene sequences are amplified and detected to identified the bacteria.

In order to choose a target molecule for sequence analysis, there are a number of considerations. The phylogenetic marker molecule needs to be i) widely distributed, ii) genetically stable, and iii) reasonably conserved and independent during evolution (Miller and Day, 2004). The 16S rRNA is usually selected as the marker. However, a recent study indicates that rRNA fails to discriminate between closely related strains in some cases and β-subunit of RNA polymerase, \textit{ropB}, has been suggested as an alternative marker molecule (Mollet \textit{et al.}, 1997). Using \textit{ropB} allows a finer degree of resolution for distinguishing among strands of bacteria, and phylogenetic trees based on \textit{ropB} are more compatible with the accepted view of relationships among species than were those based on 16S rRNA (Fox \textit{et al.}, 1992). Therefore, in our study, the probes are designed to be complementary to the \textit{ropB} sequence of the bacteria.

PCR and sequencing methods require high-cost and large size instrumentation, and culturing is still essential, which limits their application in hospitals. Therefore, the development of a rapid,
A sensitive, cost-effective, and portable bacterial speciation sensor is necessary for clinical application and environmental monitoring. Herein, we report an assembly of bacterial detection device which includes the sample preparation and detection unit to perform sample-to-answer analysis in 30 minutes (Figure 4.1). The bacteria were lysed within a microscale channel by application of an electrical field. The crude lysates were tested directly by the sensing chip with multiple microelectrodes developed in our laboratory (Fang et al., 2009; Soleymani et al., 2009a; 2009b; 2009c). Electrochemical signals were read out and analyzed by the computer.

Figure 4.1. Schematic illustration of the sample-to-answer bacterial detection device. Bacteria were lysed by applying an electrical field within a microscale channel. Lysates were transported onto the sensing chip without further manipulation or workup. Electrochemical signals were read out after incubation.
2 Experimental section

2.1 Chemicals and materials

Tin(II) chloride (anhydrous, ≥99.99%), trifluoroacetic acid (TFA, ≥98%), silver nitrate (≥99.0%), sodium sulfite (≥98.0%), formaldehyde solution (37 wt. % in H₂O), 1,1’-carbonyldiimidizole, 1,6-hexamethylenediamine, 1,4-dioxane (anhydrous, 99.8%), 3-(2-Pyridyl dithio)propionic acid N-hydroxysuccinimide ester (SPDP), dithiothreitol (DTT), dimethylformamide, m-cresol, triisopropylsilane (TIPS), 6-mercapto-1-hexanol (MCH, 97%), hexaamineruthenium chloride, potassium ferricyanide and potassium ferrocyanide trihydrate were purchased from Sigma-Aldrich Canada Ltd. DNA oligonucleotides were obtained from the Centre for Applied Genomics in the Hospital for Sick Children (Toronto, Canada).

2.2 Electrochemical measurements

Electrocatalytic currents were measured when signals reach stable in solutions of 10 μM Ru(NH₃)₆³⁺ and 4 mM Fe(CN)₆³⁻ in 25 mM sodium phosphate (pH 7), and 25 mM NaCl (any deviations from these conditions are described in individual figure captions.) Stable signals were obtained after 2 scans, and thus the second scan was used for quantitiation in all experiments.

Differential pulse voltammetry (DPV) signals were measured with a Bioanalytical Systems Epsilon potentiostat using a potential step of 5 mV, pulse width of 50 ms, pulse period of 100 ms, pulse amplitude of 50 mV; these conditions are equivalent to a scan rate of 50 mV/s. A three-electrode configuration was used consisting of a modified gold working nanowire electrode, a platinum wire auxiliary electrode, and an Ag/AgCl reference electrode. Cyclic voltammetry measurements were conducted at room temperature at a scan rate of 100 mV/s. Limiting reductive current (I) was quantified by subtracting the background at 0 mV from the cathodic current at -300 mV in a cyclic voltammetry signal. Signal changes corresponding to hybridization were calculated as follows ΔI (%) = [(I₈₅-I₈₃) / I₈₃] × 100%] (ss = before hybridization, ds = after hybridization).

2.3 Preparation and purification of oligonucleotides

The following probe and target sequences were used in experiments employing synthetic oligonucleotides.
DP-EC (DNA probe for *E. coli*):

\[ 5' \text{ATC TGC TCT GTG GTG TAG TT} 3' \]

DP-SS (DNA probe for *S. saprophyticus*):

\[ 5' \text{AAG TAA GAC ATT GAT GCA AT} 3' \]

PP-EC (PNA-AA probe for *E. coli*):

\[ \text{NH}_2\text{-Cys-Gly-Asp- ATC TGC TCT GTG GTG TAG TT -Asp-CONH}_2 \]

PP-SS (PNA-AA probe for *S. saprophyticus*):

\[ \text{NH}_2\text{-Cys-Gly-Asp- AAG TAA GAC ATT GAT GCA AT -Asp-CONH}_2 \]

DT-EC (DNA target of *E. coli*):

\[ 5' \text{AAC TAC ACC ACA GAG CAG AT} 3' \]

DT-SS (DNA target of *S. saprophyticus*):

\[ 5' \text{ATT GCA TCA ATG TCT TAC TT} 3' \]

DNA was prepared and purified as described in Chapter 2 - 2.4.

PNA probes were synthesized following the procedure described in Chapter 2 - 2.4. Two negatively charged aspartic acids were added at the terminus of the PNA, one at each end (Figure 4.2). The molecular weight of all PNA probes were confirmed by mass-spec (Figures 4.3, 4.4)
Figure 4.2. Synthesis of PNA-AA oligonucleotides for deposition. (a) HATU, N-methylmorpholine in DMF, 3 hours; (b) 20% piperidine in DMF for 10 minutes; (c) HATU, N-methylmorpholine in DMF, 3 hours; (d) repeat of step (b) and step (c); (e) addition of aspartic
acid, glycine and cysteine; (f) 85% TFA, 10% m-cresol, 2.5% TIPS, 2.5% H₂O, room temperature, 5 hours.
Figure 4.3. Mass spectrum of PP-EC. The molecular weight of PP-EC is 5855 g/mol, which matched the peak in mass spectrum, indicating the synthesis of PP-EC probe was successful.
Figure 4.4. Mass spectrum of PP-SS. The molecular weight of PP-SS is 5884 g/mol, which matched the peak in mass spectrum, indicating the synthesis of PP-SS probe was successful.
2.4 Modification of HNMEs with probe sequences for electrochemical measurements

A solution containing 1 \( \mu \)M thiolated PNA probe and 50 mM sodium chloride was deposited on the HNMEs in a dark humidity chamber overnight at room temperature. The probe was deposited using a manual micropipettor. The chips were rinsed in 25 mM sodium phosphate (pH 7), and 25 mM NaCl buffer before measurements.

Single-stranded thiolated DNA probes were immobilized on nanowire electrodes in solution containing 5 \( \mu \)M SH-DNA, 500 nM MCH, 25 mM sodium phosphate (pH 7), 25 mM NaCl and 50 mM MgCl\(_2\) in a dark humidity chamber at room temperature for 15 min. To denature the single-stranded thiolated PNA probes before immobilization, they were heated at 55°C for 10 min. They were then applied onto nanowire electrodes in solution containing 10 \( \mu \)M SH-PNA, 1 \( \mu \)M MCH, 25 mM sodium phosphate (pH 7), and 25 mM NaCl in a dark humidity chamber at room temperature overnight. Following deposition, electrodes were rinsed in 25 mM sodium phosphate (pH 7), 25 mM NaCl buffer. MCH is added in the immobilization solution to i) achieve an optimal probe film density, and ii) reduce the non-specific adsorption. The adsorption of DNA on the nanowires was confirmed by monitoring the attenuation of the electrochemical signal obtained from a solution of 2 mM ferrocyanide in 25 mM sodium phosphate (pH 7) and 25 mM NaCl solution (data not shown).

2.5 Electrochemical detection of target hybridization

The electrocatalytic current obtained from nanowire electrodes modified with thiolated probe was measured as described above, the rinsed nanowire electrodes were then exposed to target sequences, and then hybridization was detected through enhancement of the electrocatalytic signal. Hybridization solutions typically contained target sequences in 25 mM sodium phosphate (pH 7), and 25 mM NaCl. 100 mM MgCl\(_2\) were added during hybridization when DNA probes were used. Electrodes were incubated at 37°C in a thermostatted humidity chamber in dark and were washed extensively with buffer before electrochemical analysis. The conditions used for individual experiments varied depending on the size and source of the target nucleic acid; details of different hybridization trials are provided in the figure captions.
2.6 Hybridization protocol
Hybridization solutions typically contained target sequences in 25 mM sodium phosphate (pH 7), and 25 mM NaCl. 100 mM MgCl₂ were added during hybridization when DNA probes were used. Electrodes were incubated at 37 °C in a dark humidity chamber in dark for 30 min and were washed extensively with buffer before electrochemical analysis.

2.7 Kinetic Measurements of DNA hybridization at NME
PNA (seq. 1)-modified NMEs were prepared as described above. Rinsed NMEs were immersed in a solution containing 10 μM Ru(NH₃)₆³⁺, 4 mM Fe(CN)₆³⁻, 100 fM DNA target in 25 mM sodium phosphate (pH 7), and 25 mM NaCl buffer. The electrocatalytic CV signals were obtained as described above. All measurements were performed at 37 °C.

2.8 Growth and lysis of bacterial samples
Samples of *E. coli* and *Staphylococcus saprophyticus* were grown in Lysogeny Broth (LB-Broth) at 37 °C for 12 hours, where 10 mL of LB-Broth was inoculated with 1 μL of bacterial stock solution. After growth overnight, the bacterial solution was monitored by UV-Vis spectrometer to measure the absorbance at 600 nm. The absorption should be approximately 1.0. Then the samples were centrifuged at 3000 rpm for 5 min. The supernatant was discarded and replaced with an equal amount of 1× PBS (pH 7.4). Bacteria were resuspended by vortexing and then centrifuged at 3000 rpm for 5 min. The supernatant was discard and replaced with an equal amount of 1× PBS (pH 7.4). After vortexing, the bacterial samples were injected into a micro/nanochannel device (Wang et al., 2006a). Electrical lysis was achieved using a flow rate of 10 μL/min with a syringe pump, and application of 500 V. Immediately following lysis, samples were frozen and stored at -80 °C and thawed on ice when needed for hybridization. 100 μL of unlyzed and diluted bacterial sample was incubated on LB-Agar plate at 37 °C for 12 hours. Visible colonies were counted.

3 Results and discussion
3.1 Investigation of relations between sensor size and sensitivity
Nanostructures of a range of materials and assays have been used in bioassays by many research groups in order to improve the detection limits. However, the application of nanostructuring may
also come with some limitations. The required detection time could be unacceptably long when these devices are presented in a solution containing low levels of large and sluggish biomolecules, such as RNA molecules, in clinically relevant samples. As a chip-based sensor for clinical use, rapid and sensitive detection should be achieved in parallel. According to the model simulated by Leyla Soleymani and Brian Lam in our group, the relationship between sensor size and detection time is shown in Figure 4.5. Herein, we set the bacteria level at 100 μL of 1 cfu/μL, which is highly relevant to disease diagnosis in hospital, and used RNA polymerase β mRNA transcript (rpoB), a 4000 nt RNA with a copy number of approximately 1400 per cell as the molecular target, the concentration of which is approximately 2 fM.

According to the simulation results (Figure 4.5), when a 100 nm sensor was used, it takes 24 days of incubation at room temperature to guarantee one target molecule to accumulate on the sensor surface, because of the molecular transport problem reported previously in the literature (Sheehan and Whitman, 2005; Nair and Alam, 2008; Squires et al., 2008). When a target molecule in smaller size, such as a 20 bp synthetic DNA molecule, was used, the detection time is shorten to 4.8 hours on the 100 nm sensor due to the faster transportation rate. The different detection time required with target molecule of different sizes may explain the failure of some bioassays which were robust in detection of small synthetic targets but failed to detect large clinically relevant biomolecules.

In contrast, when a larger sensor was used, such as a sensor with a footprint of 100 μm, significant accumulation of a 4000 nt biomolecule target is possible within 30 minutes.
Figure 4.5. Analysis of structure size versus time required to accumulate one molecule in a solution containing 15 fM of a nucleic acid target. This concentration represents the amount of a mRNA target in a 100 μL solution containing 100 bacteria, which is a clinically relevant detection level. Accumulation times were calculated from published models. (Models were simulated by Leyla Soleymani and Brian Lam.)

3.2 Investigation of nanotextures on hierarchical microelectrodes

Previous work in our group indicated that nanoscale roughness on the electrode surface can significantly improve the detection limit (Soleymani et al., 2009c). While the ~100 μm sensors were substantially smooth on nanoscale (Figure 4.6A), nanotextures were introduced on these structures in the hope of further improve sensitivity. A thin palladium (Pd) layer was coated on the gold microelectrode using electrodeposition, as Pd is particularly versatile in producing different nanostructured morphologies depending on electroplating conditions (Soleymani et al., 2009a). After Pd coating, a layer of nanotextures ranging from 10 -50 nm was generated on the electrode surface (Figure 4.6B), and these hierarchical nanotextured microelectrodes (HNMEs) were used in this project.
Figure 4.6. Nanoscale morphology of Au and Au/Pd microstructures and generation of HNMEs. Microstructures made from Au alone (A) did not exhibit significant nanoscale roughness. Only with electrodeposition of a fine layer of Pd did we introduce nanoscale roughness on the length scale of 10-50 nm (B). (SEM images were adapted from Leyla Soleymani.)
Further research indicated that with the nanotextures, the probe surface coverage and hybridization efficiency of the synthetic DNA target were improved significantly (Figure 4.7 and Bin et al., 2010).

Figure 4.7. Hybridization efficiency of the DNA probe – DNA target duplex on Au and Au/Pd HNMEs, measured using chronocoulometry. (Research performed and data analyzed by Xiaomin Bin.)

3.3 Investigation of the effect of PNA-amino acid hybrid probes

PNA probes used in previous studies (Figure 4.8A) were charge-neutral. The background signals were suppressed due to the uncharged pseudopeptide backbone (Fang and Kelley, 2009).

However, the uncharged PNA molecules have some drawbacks. The solubilities of the PNA probes, which are related to the sequence, are generally low in aqueous solution and for some sequences, precipitation from the solution could be observed. There is a large amount of hydrogen bonding donors and acceptors on the probe structure, so that the PNAs are prone to form aggregations.
In order to solve these problems, two negatively charged amino acids, aspartic acids, were added at the terminus of the PNA, one at each end (Figure 4.8B). By making the PNA-amino acid (PNA-AA) hybrid probes slightly negative-charged, we obtained better solubilities and less secondary structures for all PNA probes with different sequences. Research performed by Elizaveta Vasilyeva in our group suggested that the thiol group on the PNA-AA probe was more reactive than that on the PNA probe.

![Figure 4.8. Comparison of uncharged the PNA probe (A) and the PNA-AA hybrid probe (B).](image)

In the assay, the electrocatalytic reporter system was used, in which the background signals could be suppressed if the probes are uncharged (Lapierre et al., 2003; Fang and Kelley, 2009). The addition of two aspartic acids on the PNA probes still showed low background signals.

### 3.4 Detection of bacterial samples in crude lysates

The bacteria were lysed within a microscale channel by application of an electrical field. The lysates were directly transferred onto the multiplexed chip sensors developed in our laboratory for analysis (Fang et al., 2009; Soleymani et al., 2009a; 2009b; 2009c). An electrocatalytic reporter system also developed in our laboratory was used to obtained electrochemical signals after sample incubation (Lapierre et al., 2003; Gasparac et al., 2004; Lapierre-Devlin et al., 2005).
A sequence of $rpoB$ from *S. saprophyticus* (*Staph*) is selected as the target, which is usually implicated with urinary tract infection. A sequence of $rpoB$ from *E. coli* is used as the negative control.

A typical clinical sample contains about 100 bacteria. In order to detect *Staph* without culture, the bioassay has to achieve the target sensitivity of 15 fM. According to the model simulation, the sensor footprint needs to reach 100 $\mu$m. Therefore, HNMEs are applied in this project.

Under optimal conditions, as few as 25 cfu *Staph* sample was detected successfully, compared to the control, which used $10^5$ cfu *E. coli* sample and the non-complementary probe (Figure 4.9).

Figure 4.9. Detection of 25 colonies of *Staphylococcus saprophyticus* (*Staph*) present as unpurified lysates. Au/Pd HNMEs were modified with PNA probes (PP-EC and PP-SS). These sensors were challenged with lysate solutions and readout within 30 minutes using an electrocatalytic reporter system. The bacterial counts correspond to the number of bacterial cells
in a 25 μL solution. Data represent average ΔI values and standard errors obtained from at least four trials.

### 3.5 Real time monitoring of bacterial samples

In order to detect the genome sequence of different species of antibiotic resistant bacteria on the HNMEs in a short time period, the hybridization kinetics must be rapid and the selectivity of the assay must be high enough to discriminate similar sequences.

100 cfu/μL *Staph* sample was monitored in real time. The PNA probe that is complementary to *Staph* showed rapid hybridization in the first 30 minutes, while the hybridization signal of the probe complementary to *E. coli*, used as the control, remained as low as the background (Figure 4.10).
Figure 4.10. Hybridization kinetics of two different bacteria lysates on HNMEs modified PNA probe PP-SS. Hybridization signals of 100 cfu/μL were monitored using Ru(III)/Fe(III) electrocatalytic reporter system. The hybridization signal for fully complementary *Staph* target increased rapidly, compared to the *E. coli* target. Error bars shown represent standard errors calculated from at least four trials.

3.6 Detection of bacterial mRNA in urine samples

The urinary tract infection (UTI) is one of the most common diseases in medical practice. It has been estimated that approximately 20-30% of women experience UTI at least once in their lifetime (Kunin, 1994). It is also the most common infections in hospital, at a rate of 2 - 3 per 100 admissions (Krieger et al., 1983).

Routine screening of hospital patients for UTI is typically done with samples that contain pathogenic bacteria at levels above the limit of detection reported (von Eiff et al., 2002; Wang et
al., 2006a). To explore the applicability of the sensors for the analysis of clinical samples, we tested neat urine samples containing either *E. coli* or *S. saprophyticus*, which are the main etiologic agents.

The level bacteria in neat urine sample should be low enough to avoid interference of the spiking bacterial samples. As shown in Figure 4.11, the number of bacteria in neat urine sample was approximately 0.5 cfu/μL, which is negligible.

Figure 4.11. Bacterial culturing from 100 μL neat urine sample. The bacteria level in urine sample is approximately 0.5 cfu/μL. Compared to the concentration of bacteria in UTI, it is negligible.
As shown in Figure 4.12, we successfully identified both pathogens in the presence of this biological background. These experiments were done with bacterial levels at 250 cfu, which is 10-fold lower than those required for the diagnosis of UTIs. The detection time is 30 minutes and no bacterial culturing is involved.

Figure 4.12. Direct detection of *E. coli* and *S. saprophyticus* (*Staph*) present in urine at concentration of 10 cfu/μL. Lysates were generated directly from spiked urine samples and applied directly to chip for 30 min before electrochemical analysis. A control probe was used in each trial to assess background signals. A current value was collected for each trial; each data point is plotted individually.

Moreover, the urine samples were lysed and analyzed on-chip with no sample preparation nor purification, which is a major advantage of this system over methods like PCR or DNA sequencing which require substantial sample purification and manipulation.
4 Conclusions

In summary, the chip-based sensors, combining i) microtechnology to eliminate the barrier of transport behavior of large biomolecules; ii) nanotechnology to improve the accessibility of the targets, and iii) integration of bacterial lysis and analysis units, exhibit robust and specific detection of bacterial species for sample-to-answer in 30 minutes without a need for sample purification or labeling.

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Chapter 5
Summary and future directions

1 Summary

Over the last two decades, due to the great advances in biotechnology, genomic analysis has become one of the most important tools in disease diagnosis. Many types of cancers, such as leukemia, prostate cancer, breast cancer, and head and neck cancer, have been reported to be related to genetic defects (Rabbitts, 1994; Tomlins et al., 2005; Mitelman et al., 2007; Persson et al., 2009). Genomic analysis is also needed in other cases, such as warfarin dosing, urinary tract infection diagnosis, tuberculosis diagnosis, or antibiotic-resistant bacteria identification. Biomarkers, such as DNA and RNA sequences, are the main targets. However, the major application of conventionally available methods to detect these biomarkers, such as PCR, fluorescent microarray, and DNA sequencing, are limited to laboratories and research institutes (Brown and Botstein, 1999; Lipshutz et al., 1999). The reasons for holding these methods from clinical application include: i) insufficient sensitivity and specificity, ii) expensive instrumentations, iii) long detection time, and iv) expertise and training required.

New diagnostic tools for genetic analysis that are rapid, sensitive, cost-effective and easy-to-use are needed urgently. Chip-based sensors are particularly suitable as a platform with all of these characteristics. For readout system, electrochemistry showed great advantages over mass readout or optical readout methods, because of the signal amplification capability, low cost, non-labeling and easy-to-conduct procedure.

In this thesis, we introduce three types of electrochemical biosensing devices combing nanotechnology and microtechnology and their applications for detection of biomarkers in clinical samples.

1. Nanoelectrode ensembles (NEEs) were able to detect prostate cancer-related gene fusions in tumor tissue samples. The detection required only 100 ng of RNA and 60-minute incubation. No enzymatic amplification is involved (Fang et al., 2009).

2. Nanostructured microelectrodes (NMEs) were developed as a chip-based and multiplexed platform, and could successfully detect multiple gene fusions simultaneously with 10 ng of mRNA and 60-minute incubation time (Fang et al., 2009; Soleymani et al., 2009a;
2009b; 2009c). Real time monitoring of target hybridization was also achieved (Soleymani et al., 2009a)

3. Hierarchical nanostructure microelectrodes (HNMEs), integrated with a bacterial lysis device, could be used as a sample-to-answer device for rapid and sensitive bacteria speciation. Detection limit was improved down to 250 cfu bacteria in neat urine samples, which was 10-fold lower than the levels in urinary tract infections. The device could also be used for bacterial monitoring in real time.

The performance of these three platforms indicated that the development of bioassays with electrochemistry, nano- and microtechnology could be extended to the detection of various cancers, genetic, and infectious diseases.

2 Future directions

The commercialization of blood glucose meters has been one of the most successful examples of bioassays using electrochemical technologies for biomarker detection since 1990s. The rapid and low cost device for point-of-care testing has improved the medical diagnosis significantly. The chip-based sensors described above could also be developed as a novel diagnostic tool for nucleic acid detection. Because of the sequence independence of the approach, probes with various sequences could be designed to screen the sample for different targets. Multiple DNA or RNA strands extracted from cancer cells, bacteria, virus, and other sources could be analyzed directly without further amplification or purification.

In the battle against diseases, new targets for bioassays are emerging everyday. For instance, the widespread use of antibiotics around the world accelerates the gene mutation rate in bacteria significantly, resulting in different strains of antibiotic-resistant bacteria (Amyes, 2001). The bacteria called “superbugs” carrying several resistance genes can survive after rounds of different antibiotic treatment. While the rate of bacterial gene mutations overtakes the development of new antibiotics, it is critical to screen the bacteria strain for its resistance before delivering any treatment, so that serious infection and the spread of bacteria could be avoided. The recent spread of bacteria expressing the New Delhi Metallo-bata-lactamase (NDM-1) enzyme from gene bla_{NDM-1} is a new generation of superbug (Kumarasamy et al., 2009; Yong et al., 2009; Payal et al., 2010) and has become a huge challenge for medical diagnosis and therapy.
It is safe to predict that more and more superbugs with various resistance genes will emerge in the future. New devices for efficient genetic detection are always demanded, since the battle between diseases and diagnosis is endless.
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