ACCOUNTS of chemical research

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Ultrasensitive Electrochemical Biomolecular Detection Using Nanostructured Microelectrodes

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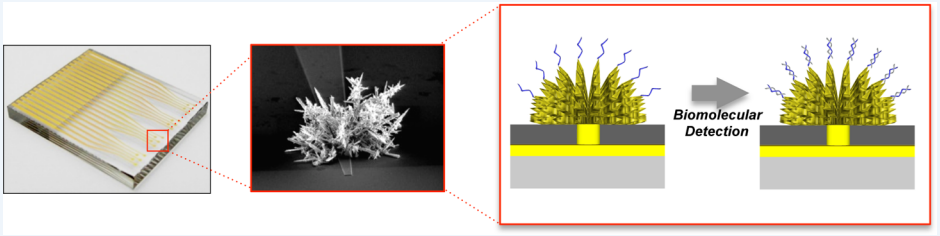


CONSPECTUS: Electrochemical sensors have the potential to achieve sensitive, specific, and low-cost detection of biomolecules—a capability that is ever more relevant to the diagnosis and monitored treatment of disease. The development of devices for clinical diagnostics based on electrochemical detection could provide a powerful solution for the routine use of biomarkers in patient treatment and monitoring and may overcome the many issues created by current methods, including the long sample-to-answer times, high cost, and limited prospects for lab-free use of traditional polymerase chain reaction, microarrays, and gene-sequencing technologies. In this Account, we summarize the advances in electrochemical biomolecular detection, focusing on a new and integrated platform that exploits the bottom-up fabrication of multiplexed electrochemical sensors composed of electrodeposited noble metals. We trace the evolution of these sensors from gold nanoelectrode ensembles to nanostructured microelectrodes (NMEs) and discuss the effects of surface morphology and size on assay performance. The development of a novel electrocatalytic assay based on Ru^{3+} adsorption and Fe^{3+} amplification at the electrode surface as a means to enable ultrasensitive analyte detection is discussed. Electrochemical measurements of changes in hybridization events at the electrode surface are performed using a simple potentiostat, which enables integration into a portable, cost-effective device. We summarize the strategies for proximal sample processing and detection in addition to those that enable high degrees of sensor multiplexing capable of measuring 100 different analytes on a single chip. By evaluating the cost and performance of various sensor substrates, we explore the development of practical lab-on-a-chip prototype devices. By functionalizing the NMEs with capture probes specific to nucleic acid, small molecule, and protein targets, we can successfully detect a wide variety of analytes at clinically relevant concentrations and speeds. Using this platform, we have achieved attomolar detection levels of nucleic acids with overall assay times as short as 2 min. We also describe the adaptation of the sensing platform to allow for the measurement of uncharged analytes—a challenge for reporter systems that rely on the charge of an analyte. Furthermore, the capabilities of this system have been applied to address the many current and important clinical challenges involving the detection of pathogenic species, including both bacterial and viral infections and cancer biomarkers. This novel electrochemical platform, which achieves large molecular-to-electrical amplification by means of its unique redox-cycling readout strategy combined with rapid and efficient analyte capture that is aided by nanostructured microelectrodes, achieves excellent specificity and sensitivity in clinical samples in which analytes are present at low concentrations in complex matrices.

■ INTRODUCTION

Diagnostic testing for disease requires analytical methods with a high degree of sensitivity and specificity. Methods that meet these requirements have been developed for research and centralized clinical laboratories, in which expert operators and the batching of patient samples together allow for complex sample processing and the use of costly analysis infrastructure.

Translating molecular diagnostics closer to the point-of-need—the doctor's office, the pharmacy, the surgical theater, the remote clinic in the developing world—will demand that further, even more stringent, requirements be met. Specifically, bioanalysis platforms will need to retain high performance while adding speed, portability, and cost-effectiveness.

The pursuit of electrochemical systems for biomolecular analysis has received significant attention over the last two decades. The commercial and clinical success of electrochemical glucose sensors[1] hinted that analogous systems could

Received: March 26, 2014
Published: June 25, 2014

   dx.doi.org/10.1021/ar500130m | Acc. Chem. Res. 2014, 47, 2417−2425

be developed for the analysis of a vast range of biomolecular analytes of interest. While this was an inspiring example of a compelling success for an electrochemistry-driven, portable, and low-cost solution, it was also clear that significant further innovation would be required to make the same capability applicable to other analytes. The electrochemical detection of glucose harnesses a natural pathway that is inherently linked to a redox reaction. In contrast, the detection of nucleic acids, non-redox-active proteins, and small molecules would require a strategy to produce a change in electrochemical current related directly to the binding of specific analytes.

Over the last two decades, a variety of electrochemical detection systems have been developed to meet this challenge (Table 1). Approaches to electrochemical biomolecular detection have included sandwich-type assays to deliver electrochemically active groups,[2,3] assays detecting conformational changes in molecular-beacon-like DNA structures,[4−9] tetrahedral DNA nanostructures,[10] enzymatic labels that generate amplified currents,[11,12] bioelectrochemical switches,[13−15] biobarcode assays,[16,17] biodiscs,[18] and non-covalent reporter systems.[19,20] In addition, numerous electrochemistry-based platforms have been developed to monitor various analytes, including drugs,[21] enzymatic activity,[22] microRNAs,[10] metabolites,[23,24] and proteins.[25] Despite so many exciting developments in this field, only one electrochemical analysis system is available for clinical use.[26] This platform is based on the dual hybridization of an amplified gene target to an electroactive ferrocene-labeled probe and an additional capture probe immobilized on a gold electrode. As a result of this system's moderate sensitivity, it depends on an additional sample preparation step in which the target molecules are amplified by polymerase chain reaction (PCR), thus limiting the ability of this device to provide short sample-to-answer times.

Table 1. Electrochemical Detection Strategies

detection strategy	analyte	detection limit	ref
biobarcode assay	DNA	4.2 fM	17
enzymatic labels	cocaine	3.4 μM	12
non-covalent reporter	ssDNA	10 aM	31
bioelectrochemical switches	antibody	0.3 nM	13
DNA nanostructures	microRNA	1 fM	10
sandwich assay	DNA	10 fM	2

In this Account, we describe our efforts to develop and characterize rapid and sensitive electrochemical sensors that can be used to detect a diverse range of targets (Table 2) in a direct manner without reliance on molecular amplification. A key development was the fabrication of highly nanostructured microelectrodes that exhibit very high levels of analytical sensitivity. We describe our efforts to validate these sensors using target biomarkers that are important for the diagnosis of cancer and infectious disease. The coupling of these devices with sample processing modules is also presented. We portray a suite of developments that, taken together, have enabled the rapid biosensing of analytes at concentrations that approach attomolar levels and that provide valuable diagnostic information in a matter of minutes.

Table 2. Performance of the Nanostructured Microelectrode Platform

analyte class	analyte	detection limit	ref
DNA	20 bp ssDNA	10 aM	32
small molecule	cocaine	1 μM	45
protein	CA-125	0.1 unit/mL	41
antibody	HIV antibody	1 ng/mL	44
mammalian cell	K562 cells	0.1 cells/microliter	37
bacterial RNA	*E. coli* RNA	0.4 cfu/μL	47

DEVELOPMENT OF AN ELECTROCATALYTIC ASSAY FOR NUCLEIC ACID ANALYSIS

The sensitivity, or detection limit, of a biomolecular detection method is driven by the efficiency of target capture and binding by a sensor combined with the translation of a modest change in a molecular layer into a large change in a measurable quantity such as electrical current. Achieving high levels of analytical sensitivity is predicated on reaping as much signal amplification as possible from a set of reporter groups. We hypothesized that if electrocatalysis could be leveraged within a detection assay, then the large currents generated could yield high levels of sensitivity. The initial design of the assay featured the use of the well-studied redox-active reporter hexaammineruthenium(III), $Ru(NH_3)_6^{3+}$, as the primary electron acceptor (Figure 1). Ruthenium binds nucleic acids through the phosphate backbone via electrostatic interactions[20] and accumulates at probe-modified electrodes at levels that correspond to the levels of the target analyte. While a small amount of Ru(III) binds to the probe-modified electrode prior to binding of the target, the amount of negative charge at the electrode surface increases after the target sequence binds, and the level of Ru(III) increases.

During the potential sweep of a current−voltage scan, Ru^{3+} is reduced at the electrode surface and generates a current that directly reports on the presence of DNA or RNA bound to the working electrode. Therefore, Ru^{3+} can be employed to monitor hybridization events, since the current generated by a double-stranded (ds) complex is significantly higher than that for the single-stranded (ss) probe. Although Ru^{3+} is a good electrochemical reporter, its use does not yield high levels of sensitivity because only one electron can be accepted by each Ru^{3+} acceptor. Thus, the limited concentration of Ru^{3+} is insufficient to produce detectable current under standard hybridization conditions.[20] To generate larger currents from Ru^{3+}, we introduced a second electroactive species, $Fe(CN)_6^{3-}$ as a secondary electron acceptor (Figure 1A). The negative charge of $Fe(CN)_6^{3-}$ prevents this complex from reaching the surface of the electrode because of the electrostatic repulsion by the negatively charged phosphate backbone. Thus, the Fe^{3+} complex remains in solution and acts as an oxidant when it interacts with outwardly diffusing Ru^{2+}, regenerating Ru^{3+} (Figure 1A). By regenerating Ru^{3+}, one bound ruthenium complex is able to interact with the electrode on multiple occasions, causing a significant amplification in the current recorded (Figure 1B). Lapierre et al.[20] found that the introduction of Fe^{3+} to this scheme resulted in a 10−50 fold increase in the hybridization currents at DNA-modified electrodes.

Multiple properties of a probe monolayer need to be optimized simultaneously: the ability to interact with the electrochemical reporter system and the capacity to maintain high hybridization efficiency. Taft et al.[27] studied the effects of probe linker length and structure on electrochemical reporters. They observed that differing linkers did not alter the

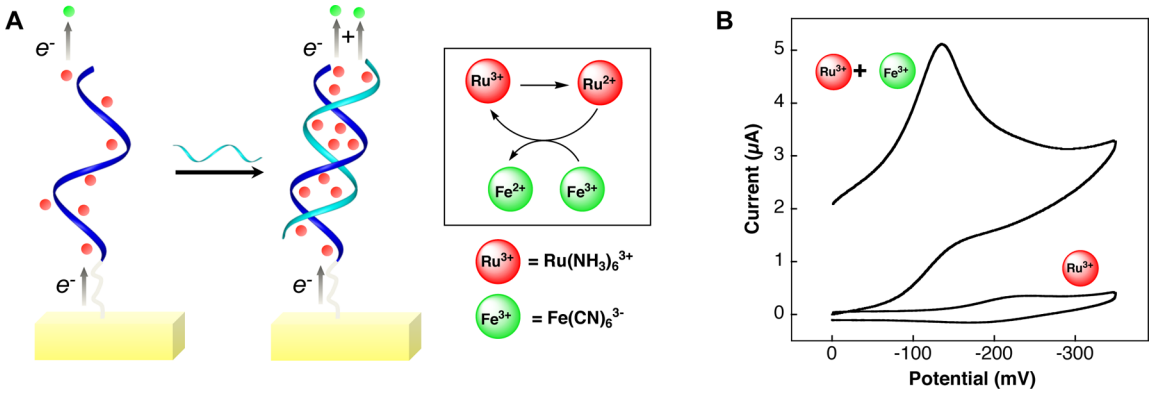


Figure 1. Ru^{3+}/Fe^{3+} reporter system. (A) The amount of Ru^{3+} (red) is greatly increased by the presence of dsDNA (light/dark blue) vs ssDNA (dark blue). Ru^{3+} (red) is regenerated by Fe^{3+} (green), completing the redox couple. (B) Cyclic voltammetry scans showing the amplification of current from the Ru^{3+} (red) and Fe^{3+} (green) electrochemical reporter system vs Ru^{3+} (red) alone. Adapted from ref 20.

electrochemical properties of the reporters and that the choice of linker did not affect the sensitivity.

Fang and Kelley[28] made a significant advance when they showed that uncharged nucleic acids provide a considerable enhancement in sensitivity. Peptide nucleic acids (PNAs) are synthetic charge-neutral compounds composed of a peptide backbone with DNA nucleobases replacing the traditional peptide side chains. PNA is able to hybridize with both DNA and RNA in classical, right-handed, double-helical complexes of Watson–Crick base pairs. Since PNA lacks the phosphate backbone, it forms more thermally stable complexes with DNA and RNA and, most importantly from an electrochemical sensing standpoint, will have little Ru^{3+} bound to an unhybridized probe. Consequently, the background signals for the Ru^{3+}/Fe^{3+} electrochemical reaction are greatly reduced. This reduction in background was found to significantly increase the overall sensitivity of the reporter system by more than 2000% (with a detection limit of 100 fM) over conventional DNA-based probes.

EFFECT OF SENSOR SURFACE MORPHOLOGY ON DETECTION PERFORMANCE

While initial work on the development of an electrochemical reporter strategy and optimization of the probe chemistry yielded promising results, the detection limits realized were still not sufficient for the analysis of most clinical samples. It was hypothesized that hybridization occurred with poor efficiencies on the surface of the gold electrodes used for detection. Indeed, the fact that the hybridization levels were much lower than those observed when the probes were in solution raised the question of whether the structure of the probe monolayer, and its resultant accessibility to analyte molecules, was optimal for target capture. The bulk gold electrodes that were being used for capture lay essentially flat on the molecular length scale. A high degree of probe–probe interaction or the use of probe densities that were too high to promote efficient binding could explain the low hybridization efficiencies.

The use of materials with nanoscale features presented a means to improve the probe presentation by providing an electrode substrate that could increase the angle of deflection among nearby probe molecules through the nanoscale curvature of the sensor surface. To test this idea, gold nanoelectrode ensembles (NEEs)[29,30] were used with the electrocatalytic assay described above.[31] Two-dimensional (2D) NEEs were created by growing 10 nm diameter nanowires in a track-etched polycarbonate membrane template, and a plasma etching process exposed the gold nanowires, which resulted in three-dimensional (3D) NEEs. The 2D and 3D NEEs were tested alongside the macroelectrodes made of bulk gold that had been used previously. Each electrode was functionalized using a nucleic acid probe complementary to the 23S rRNA gene from *Helicobacter pylori* and challenged with the corresponding ssDNA. A striking result was obtained, as the detection limits were improved by a factor of 10^6 through the use of the nanoelectrode ensembles. These showed higher peak currents than the macroelectrodes after hybridization. The 3D nanoelectrodes yielded the best performance and the highest peak currents after hybridization. The enhanced diffusion profile for 3D nanoscale sensors resulted in an amplification of the electrocatalysis, which led to high signal-to-noise ratios. With 3D NEEs, a 1 pM limit of detection was realized, which at the time was the best sensitivity achieved with an electrochemical assay for a nucleic acid sequence.

While the NEEs were an excellent platform for testing the influence of nanostructuring on the detection sensitivity, the materials chemistry involved in their fabrication made multiplexing and the fabrication of robust devices a challenge. To probe the relationship between sensor morphology and sensitivity further, we developed a new sensor platform that would allow nanostructured features to be produced reproducibly on the surface of an electrochemical sensor. Using a combination of top-down and bottom-up fabrication, we developed sensing electrodes with nanoscale features that could be rapidly fabricated and functionalized on a single planar integrated circuit (Figure 2A).[32] We then patterned gold leads on a silicon substrate using conventional photolithography. These leads served as a template for the electrodeposition of noble metals. The leads were passivated with oxide, and 500 nm apertures were exposed at the tips of the leads. Palladium, a noble metal that is easily functionalized using thiolated probes (Figure 2B), was electrodeposited into the apertures to grow nanostructured microelectrodes (NMEs).

The extent of branching of the resultant nanostructures was readily programmable by tuning the electrodeposition conditions (Figure 2C). We found that using $HClO_4$ as the supporting electrolyte resulted in 20–50 nm nanostructures, while sensors electroplated with an HCl supporting electrolyte had surface roughness on the order of 100–300 nm. HCl slows the growth of the nanostructures by inhibiting the ionization of the palladium(II) salt. This slow growth yielded smooth

dx.doi.org/10.1021/ar500130m | *Acc. Chem. Res.* 2014, 47, 2417–2425

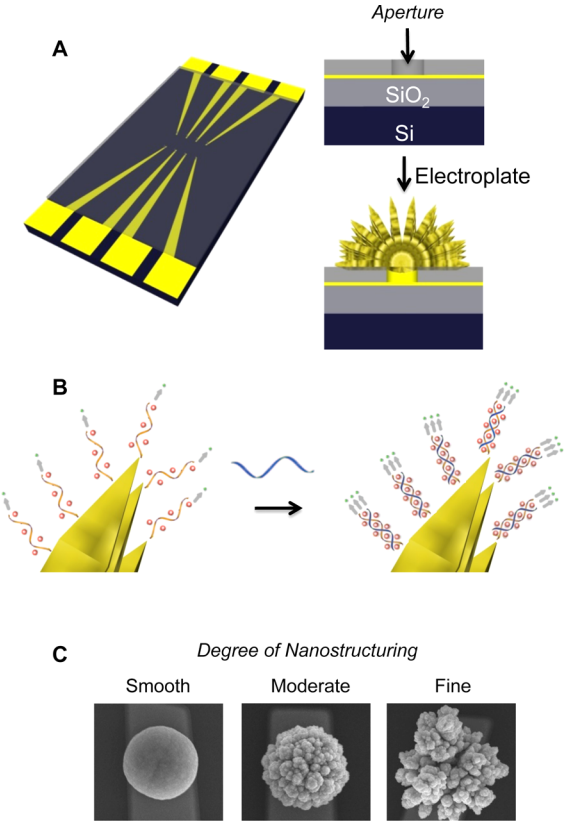


Figure 2. (A, B) Schematic illustrations of the fabrication of silicon microchips for NME assays. (A) A layer of gold is deposited on a silicon wafer using conventional photolithography. The surface is then passivated with a layer of SiO_2, creating apertures for NME deposition. NMEs are deposited using Pd or Au. (B) Probes are deposited on NMEs via a noble metal−thiol bond and can be hybridized with complementary DNA/RNA target sequences. (C) SEM images of NMEs with smooth (left), moderate (middle), and fine (right) degrees of nanostructuring. Adapted from ref 32.

structures. When electrodes were deposited in a $HClO_4$ electrolyte, the sensors grew rapidly with a highly fractal nature as $HClO_4$ does not inhibit nanostructure growth. The accelerated electrodeposition kinetics yielded branchlike protrusions.

Despite the fractal nature of the sensors, the overall electrochemically active surface area was reproducible. Using cyclic voltammetry, we found a less than 5% degree of variation between sensors in scans with $Fe(CN)_6^{4-}$ and $Ru(NH_3)_6^{3+}$. We found a similar degree of reproducibility when we studied the signal changes due to hybridization with complementary ssDNA targets.

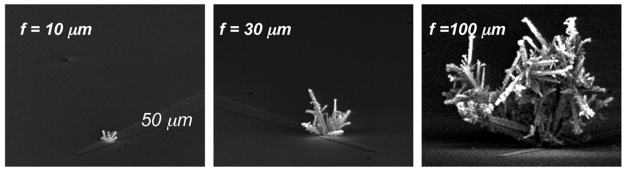
When challenged with a synthetic DNA target, sensors with a greater degree of nanostructuring were found to be more sensitive. Smooth sensors had a 100 fM detection limit, while sensors with 20−50 nm nanostructuring had a detection limit of 10 aM. Using a combination of three sensors with different levels of nanostructuring, we achieved six orders of dynamic range in the limit of detection.

We also studied the effect of nanostructuring on the hybridization efficiency and probe surface coverage. Using chronocoulometry, we extracted the probe coverage and hybridization efficiency on sensors with varying degrees of nanostructuring.[33] We found that sensors with finer nanostructures had higher probe coverage than smooth sensors. This result matches an effect obtained with gold nanoparticles that suggested that a high radius of curvature promotes dense probe packing on nanomaterials because of the opportunity for an angle of deflection to be created between probes.[34] Nanostructuring was found to increase the efficiency of hybridization by several orders of magnitude compared with the case of smooth sensors. Thus, by increasing the deflection angle of the molecules on the nanorough surface, we were able to achieve an efficient display of probe molecules on the nanostructured sensor, resulting in a significant improvement in efficiency.

EFFECT OF SENSOR SIZE ON DETECTION PERFORMANCE

The performance achieved with the NMEs was impressive and indicated that clinically relevant levels of nucleic acids could be analyzed with this approach. However, analyzing gene expression in clinical samples demands the sensing of large messenger RNAs (mRNAs), leading to a new challenge. The slow diffusion of large analytes necessitates impractical wait times to ensure that a sufficient number of these molecules reach the sensor surface. To illustrate the challenge of detecting large analytes, we performed calculations on the hybridization times that would be necessary to detect a typical 4000 base pair (bp) mRNA molecule with a copy number of 1400 at a clinically relevant concentration of 1 cfu/μL.[35] We calculated that it would take 24 days to accumulate just 1 molecule of mRNA on a 100 nm sensor but only 12 min on a 100 μm sensor.

In contrast, rapid sample-to-answer turnaround, on the order of 20−30 min, will be required to enable the delivery of rapid, cost-effective test results in physician's offices or at the patient bedside. We therefore sought to find a way to increase the interaction of the NMEs with the molecules in solution. We found that gold structures could be electrodeposited with 100 μm footprints suitable for the rapid accumulation of slowly diffusing analytes. High plating potentials and salt concentrations yielded fine nanostructures, while longer plating times yielded sensors with larger footprints (Figure 3). As electrodeposited, these structures had a smooth surface on length scales shorter than 100 nm. We therefore introduced a Pd



Sensor footprint	10 μm	30 μm	100 μm	10 μm	30 μm	100 μm
cells/μl	1.5	1.5	1.5	150	150	150
# of molecules in 30 min	< 1	< 1	1	9	34	186
Successful detection	✗	✗	✓	✓	✓	✓

Figure 3. Hierarchical nanotexured electrodes. Large, slowly diffusing analytes accumulate more rapidly on sensors with a larger footprint. In a lysate from bacteria at 1.5 cfu/μL, sensors smaller than 100 μm did not capture even a single 4000 bp transcript after 30 min. As a result, only the largest sensors (100 μm) could detect the bacteria at such low concentrations. Reprinted from ref 35. Copyright 2011 American Chemical Society.

dx.doi.org/10.1021/ar500130m | Acc. Chem. Res. 2014, 47, 2417−2425

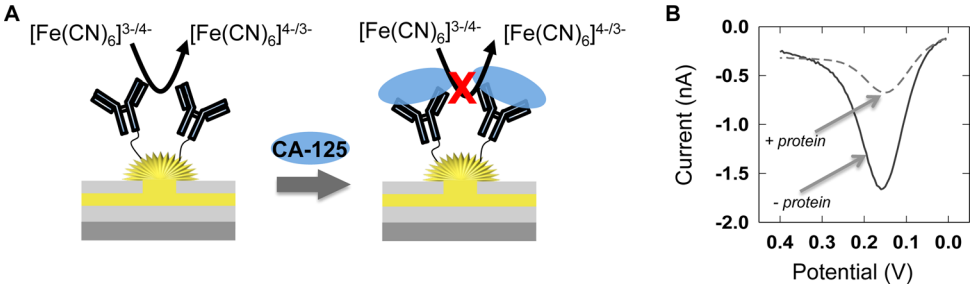


Figure 4. NME protein detection scheme. (A) Binding of the antigen (CA-125) to the antibody (black) blocks the access of $[Fe(CN)_6]^{3-/4-}$ to the electrode surface. (B) Differential pulse voltammetry scans showing the signal decrease (dashed line) observed after the addition of CA-125 at 10 units/mL in serum. Reprinted from ref 41. Copyright 2011 American Chemical Society.

overcoat to generate the fine nanostructuring necessary for high sensitivity. To illustrate the advantage provided by these multilayer electrodes, we challenged 10, 30, and 100 μm sensors with *Escherichia coli* lysate. The sensors were modified with a PNA probe complementary to *E. coli* rpoβ, a 4000 bp transcript. Only the sensors with a 100 μm footprint could detect the lowest concentration of *E. coli* at 1.5 cfu/μL within a clinically relevant time frame of 30 min.

DETECTION OF CLINICALLY RELEVANT BIOMARKERS USING NANOSTRUCTURED MICROELECTRODES

Since their initial development in 2009, NMEs have been used to detect a number of clinically relevant targets. We have analyzed cancer-related gene fusions implicated in prostate cancer and leukemia in heterogeneous samples using this approach. Gene fusions are particularly challenging to detect with a high degree of specificity because the unfused wild-type genes may also be present in the sample. Using carefully designed probes, we deployed the NME detection strategy and found it to be effective when RNA extracted from prostate tumors was analyzed[36] and when blood-borne leukemia cells were analyzed.[37] The multiplexing that can be engineered into the chip-based NME platform enabled the detection of gene fusions, allowing us to control for the presence of wild-type sequences and also to detect multiple fusion types.

MicroRNAs (miRNAs) represent another challenging target for traditional detection platforms. MicroRNAs are important biomarkers that show significant promise for applications in the clinic, with variable expression levels that may help classify cancers and more specifically identify other types of disease.[38] They are challenging to detect because of their small size, low abundance, and high levels of sequence homology (miRNAs can differ by as few as one nucleotide). We found that the NME platform was able to detect miRNA at concentrations as low as 10 aM (10 molecules/μL) within 30 min and discriminate between target miRNAs that varied by a single nucleotide.[39] This work was expanded to demonstrate that electrochemical detection of miRNAs could be performed using RNA samples isolated from head and neck squamous cancer cell lines.[39] These findings further highlighted the utility of this platform to detect a wide range of targets.

We also deployed the NME sensor system approach to study the genetic properties of circulating tumor cells (CTCs). In a pilot study using blood samples from patients with prostate cancer, significant changes in electrochemical readouts were reported for those patients with the highest Gleason scores, indicative of a more aggressive form of cancer.[40] Together, these developments in cancer diagnosis demonstrate that electrochemical sensing techniques possess superior sensitivity and specificity and have turnaround times on the scale of minutes. This rapidity of sensing compares favorably with the hours or days required for traditional diagnostics.

NEW ASSAYS FOR UNCHARGED ANALYTES

While detection of nucleic acids represents a compelling capability, the power of electrochemical detection would be enhanced further if it could be extended to the analysis of proteins and small molecules. In general, these classes of molecules are challenging to detect using conventional electrochemical assays because the detection method cannot exploit the charge of the analyte: unlike nucleic acids, which have a negatively charged backbone, many proteins and small molecules may be uncharged or weakly charged. Several alternative assays have been developed for the NME platform to increase its versatility and potential uses.

As proteins are not uniformly charged like nucleic acids, the readout system cannot be based on a change of surface charge at the electrode surface. To overcome this challenge, we developed a novel electrochemical assay for protein detection that relies on inhibition of electron transfer after the target protein binds to the sensor.[41] For these sensors, gold electrodes patterned on a silicon substrate were modified with antibodies that specifically recognize the ovarian cancer biomarker CA-125. In the absence of bound CA-125, there is efficient electron transfer between the electrode and the redox reporter, $[Fe(CN)_6]^{3-/4-}$; after binding of CA-125, electron transfer to the redox reporter system is hindered (Figure 4A). We assessed the sensitivity of our system by challenging our sensors with CA-125 in human serum and whole blood. Using differential pulse voltammetry, we measured the peak currents before and after binding of CA-125 (Figure 4B) and found a limit of detection of 0.1 unit/mL.

We adapted this assay to develop a system to count cancer cells.[42] Quantifying the number of tumor cells circulating in the bloodstream is a promising approach for diagnosis and prognosis of cancer.[43] Gold circular electrodes patterned on a glass chip were modified with anti-EpCAM antibodies that specifically recognize DU-145 cells, a prostate cancer cell line. Prior to cell binding, electron transfer to the redox reporter was uninhibited. When the device was challenged with a sample containing DU-145 cells, the DU-145 cells were specifically captured at the electrode by the immobilized anti-EpCAM antibodies. The bound cells hindered electron transfer to the redox reporter, reducing the peak current as measured with differential pulse voltammetry. By tuning the active electrode

dx.doi.org/10.1021/ar500130m | *Acc. Chem. Res.* 2014, 47, 2417−2425

area, we detected cell concentrations as low as 125 cells per sensor with 150 μm diameter sensors.

Rapid and robust methods to detect antibodies have important clinical applications for detecting infectious diseases such as HIV.[44] One limitation with electrochemical enzyme-linked immunosorbent assays (ELISAs) is that the noble-metal electrode must be functionalized with a thiol. This imposes limitations on the types of molecules that can be attached. Also, the multiple layers of molecules that must be immobilized on the electrode can hinder electron transfer.

To overcome these challenges, we developed a novel electrochemical ELISA for HIV to readout the presence of antibodies captured in close proximity to the electrode. 3D NMEs were patterned on glass, and the substrate was passivated with SU-8. HIV-1 antigens were covalently bound to SU-8, a photoresist epoxy. We challenged the device with clinical samples containing anti-HIV-1 gp41 antibodies, which are captured on the surface by the immobilized HIV-1 peptide. A secondary enzyme-linked reporter antibody, anti-IgG conjugated to alkaline phosphatase (ALP), binds to the anti-HIV-1 antibodies. ALP converts the electrochemical reporter *p*-aminophenylphosphate to *p*-aminophenol (PAP). The amount of PAP generated is rapidly read out at the 3D electrode within minutes using differential pulse voltammetry. When challenged with clinical samples, we found a detection limit of 1 ng/mL for HIV-1 and HIV-2.

Ultimately, we sought to develop a new assay that would enable the broadest range of analytes—irrespective of their state of charge—to be sensed on a convenient electrochemical platform. We developed a novel electrochemical assay, termed the neutralizer displacement assay, to detect a wide range of charged and uncharged analytes (Figure 5).[45] This new assay overcame the traditional limits of electrochemical assays and their detection of highly charged analytes alone.

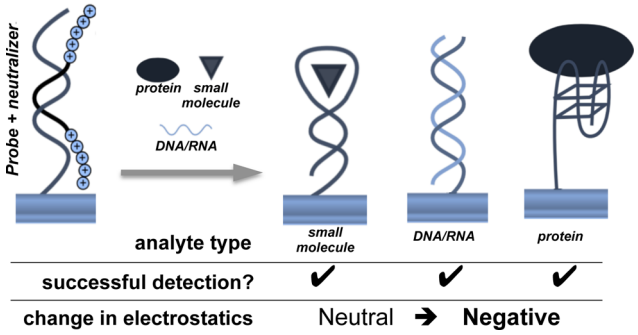


Figure 5. The neutralizer displacement assay. Detection of small molecules (gray triangles), DNA/RNA (light-blue lines), and protein (black ovals) is achievable with the neutralizer displacement assay. Bound target displaces the positively charged neutralizer (black), allowing for a significant increase in the amount of positively charged Ru^{3+} at the electrode surface. Adapted from ref 45.

In the neutralizer displacement assay, an aptamer nucleic acid probe engineered to bind to the analyte is immobilized on the surface of a nanostructured microelectrode. We designed a positively charged complementary PNA molecule that then binds to the aptamer probe. This complementary PNA strand is the neutralizer, since the positive charges neutralize the negatively charged backbone of the nucleic acid aptamer probe. The nearly neutral aptamer–neutralizer complex suppresses the background signal and allows for a high signal-to-noise ratio. The aptamer preferentially binds to the analyte because the neutralizer is designed with base-pair mismatches. In the presence of the analyte, the aptamer–neutralizer complex dissociates and the aptamer binds to the analyte. The change in charge at the sensor surface due to the dissociation of the aptamer–neutralizer complex is then readily detected using an electrocatalytic reporter assay.

As the change in signal results from dissociation of the aptamer–neutralizer complex, this assay is not restricted to charged analytes. By choosing the appropriate aptamer, we demonstrated high-sensitivity detection of a wide range of analytes, including small molecules, proteins, and nucleic acids (Table 3). As proof-of-principle detection of small molecules, we detected cocaine with a limit of detection of 1 μg/mL. We demonstrated protein detection by challenging the sensor with thrombin and found a limit of detection of 10 fM. We also challenged the system with nucleic acids. Here we used a complementary nucleic acid strand as the probe. We showed detection of *E. coli* mRNA in unpurified lysates at concentrations as low as 0.15 cfu/μL. This approach, in which the signal is generated by the dissociation of the neutralizer from the probe, is applicable to a wide class of detection schemes in which the readout is dependent on the charge at the sensor surface.

Table 3. Performance of the Neutralizer Displacement Assay

analyte	classification	limit of detection
ATP	small molecule	1 mM
cocaine	small molecule	1 μg/mL
DNA oligonucleotides	nucleic acid	100 aM
RNA	nucleic acid	10 pg/μL
E. coli lysate	nucleic acid	0.15 cfu/μL
thrombin	protein	10 fM

LAB-ON-A-CHIP DEVICES FOR POINT-OF-CARE APPLICATIONS

To realize the full translational potential of a sensor platform, there must be evidence that it can be fully automated and developed into a user-friendly device. An important consideration when developing novel diagnostics is that of sample processing. Devices that are able to function robustly in relatively crude and complex matrices are highly desirable in comparison with those that require lengthy purification steps. Integrated sample processing units often remain distinct to the detection platform.

Using an electrical lysis method, we successfully coupled a sample-processing unit to the NME chip and Ru^{3+}/Fe^{3+} reporter system (Figure 6A).[46] A bacterial sample was introduced into a lysis chamber that consisted of two gold electrodes separated by 500 μm. A 100 V pulse was applied for 20 s to lyse the bacteria in the sample and subsequently release intracellular mRNA. Following lysis, the unpurified lysate could then be directly transferred to an array of NMEs for genetic analysis. In doing so, 1 cfu/microliter of either *E. coli* or *Staphylococcus saprophyticus* was detected in 30 min. Furthermore, the system was challenged with a urine sample spiked with both *E. coli* and *S. saprophyticus* to simulate a clinical urinary tract infection. The lysis and multiplexed detection scheme was able to discriminate the two bacterial samples with readouts occurring in real time in as little as 2 min.[46] These

dx.doi.org/10.1021/ar500130m | *Acc. Chem. Res.* 2014, 47, 2417–2425

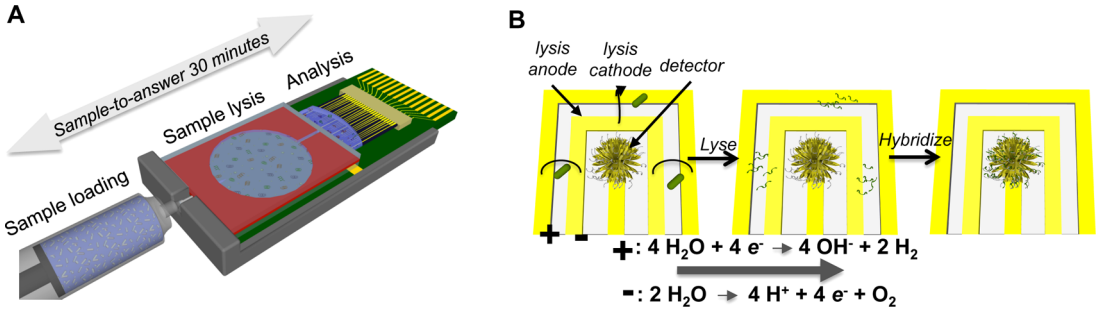


Figure 6. Integrated devices for sample preparation and detection. (A) An integrated device for electrical lysis and electrochemical readout of bacterial mRNA. The sample is first electrically lysed by application of a potential pulse. With a syringe, the lysate is transferred to NME sensors for electrochemical analysis of the mRNA. Adapted from ref 46. (B) Proximal lysis and detection of bacterial mRNA. To reduce the time required for analyte molecules to diffuse to the sensors, bacteria are electrochemically lysed in close proximity to the sensor. Released intracellular mRNA can rapidly diffuse to the adjacent NME. Reprinted from ref 47. Copyright 2013 American Chemical Society.

results clearly demonstrate the feasibility of developing the Ru^{3+}/Fe^{3+} and NME platform into a portable diagnostic device.

In a typical integrated device, bacteria are lysed in a chamber and the resulting lysate is transferred to a detection chamber for genetic analysis. If the bacteria are present at low concentrations, it can take hours or even days for a dilute solution of target mRNA molecules to diffuse to the sensor. We recognized that prior to lysis the nucleic acids are present at locally high concentrations inside the bacterium because multiple copies of a transcript exist in each bacterium. We leveraged this fact using a novel device capable of electrochemical lysis and detection where the latter process takes place proximal to the location of lysis, thereby circumventing the need to wait for diffusion over long distances to occur (Figure 6B).[47] This device consisted of an array of microwells, each of which contained gold lysis electrodes patterned within 50 μm of an NME. The sensors were functionalized with probes that recognize *E. coli* rpoβ, and the bacterial samples were then lysed in the wells by application of 20 V to the lysis electrodes. As a result, the released intracellular mRNA could rapidly diffuse to the sensor surface. We found that this electrochemical proximal lysis approach provided a 10-fold sensitivity improvement compared with off-chip lysis. Using this approach, we demonstrated that we could detect *E. coli* at concentrations as low as 0.4 cfu/μL in 2 min.

While the NME technology is amenable to multiplexing, there are limitations that arise because of the need to contact each electrode individually. We recently showed that a novel solution-based electronic switch could allow us to interrogate more than 100 different analytes using a small number, and a standard set, of electrical contacts (Figure 7).[48] Using conventional photolithography, we patterned a series of channels on a glass substrate, resulting in the formation of discrete liquid compartments. Working electrodes were then multiplexed on common leads but were electrically isolated from the reference and counter electrodes. Thus, by addressing a particular reference, working, and counter electrode combination, transient circuits were established for a single working electrode in the presence of many different working electrodes. Prior to this, each electrical sensor required an individual, independently addressed contact, and therefore, the degree of electrochemical multiplexing remained limited by package-to-chip practical considerations. By creating rows of sensors perpendicular to rows of Ru^{3+}/Fe^{3+} solutions, combined with our novel switching matrix, we were able to address individual sensors using transient solution-based circuits. This was shown to be effective in monitoring various combinations of urinary tract infections that may possess antibiotic resistance genes with detection limits of 1 cfu/μL as rapidly as 2 min.

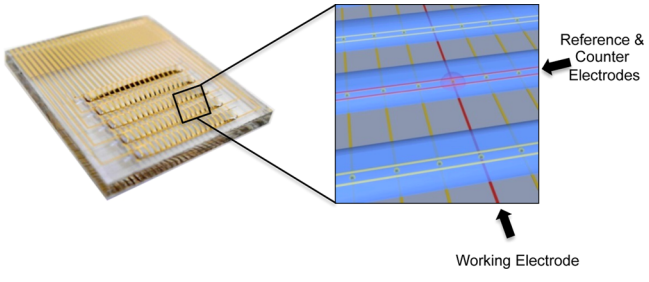


Figure 7. The solution circuit chip. The highly multiplexed NME-based microchip has five liquid channels, each containing 20 sensors. The inset shows the on-board reference and counter electrode pairs (horizontal red lines) and the working electrode (vertical red line). Adapted from ref 48.

The cost of an electrochemical biosensor represents a key consideration governing the practicality of the proposed device. We therefore explored several low-cost substrate alternatives to silicon for integrated NME growth.[49] Both printed circuit boards (PCBs) and fluorinated ethylene polymers (plastics) were shown to be cost-effective; however, they performed poorly in the ability to produce reliable NMEs, and biosensing applications were unsuccessful. Standard borosilicate glass was tested as a substrate and was found to be a good alternative to silicon that offers a similar level of fabrication and biosensing capabilities as silicon wafers but a reduced cost. By meeting the needs of a highly multiplexed, cost-effective platform, the Ru^{3+}/Fe^{3+} and NME biosensing scheme is poised for rapid technological advancement and adaptation.

■ CONCLUSIONS AND FUTURE OUTLOOK

In this Account, we have summarized advances in the development of novel ultrasensitive electrochemical assays based on nanostructured microelectrodes for a wide range of clinical applications. When coupled with an electrocatalytic reporter system, noble-metal NMEs directly report native levels of a vast array of analytes including nucleic acids, proteins, small molecules, and whole cells. They do so in the complex matrix of a variety of patient samples that include blood and urine. Recently we have developed complete point-of-care devices by integrating these NMEs into lab-on-a-chip platforms for sample processing and analysis.

dx.doi.org/10.1021/ar500130m | *Acc. Chem. Res.* 2014, 47, 2417−2425

Moving forward, there are several areas of study that will enhance the nanostructured sensing platform. Investigations into the storage and stability of the sensors will allow for the determination the optimal protocols for maintaining the long-term integrity of the functionalized biosensors. In addition, the development of novel strategies for the detection of small peptides and proteins with no known aptamers will produce diagnostic applications in additional therapeutic areas.

A remaining challenge is the rapid detection of rare cells and molecules in blood. This class of problem arises in many applications, including the detection of rare circulating tumor cells, circulating fetal DNA in maternal blood, and infectious pathogens in bloodstream infections. This problem is particularly daunting, as it demands a device with extraordinary sensitivity and specificity. In the case of circulating tumor cells, the cells of interest are present at concentrations of just 1−100 cells/mL among a background of billions of red blood cells and millions of white blood cells. No existing sensor can detect native levels of nucleic acids at such low concentrations among such a confounding background. Thus, these applications require a preconcentration step to isolate the cells of interest from the background. Recently there have been many promising advances using microfluidics for capture and isolation of circulating tumor cells. Therefore, research efforts to couple these preconcentration steps with sensors onto a single integrated chip are highly desirable.

The detection of rare analytes raises a challenge beyond sensitivity and specificity—that of throughput. At levels of just a few cells per milliliter, approximately 10 mL must be sampled to increase the likelihood of capturing a sufficient number of cells for detection. However, many current sensing platforms are designed to work with microliter volumes, and most integrated devices for sample preparation are built using microfluidics. These devices, by their microscale nature, are not usually amenable for processing of large volumes in short turnaround times. Efforts to increase the throughput of integrated devices are urgently needed to meet the clinical need for rapid turnaround times.

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Notes

The authors declare the following competing financial interest(s): S.O.K. and E.H.S. own equity in Xagenic Inc., a company commercializing an electrochemical diagnostic platform.

Biographies

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Brian Lam was born in Toronto, Canada, in 1982. He received a B.Sc. in Engineering Science from the University of Toronto and then received his Ph.D. in Chemistry from the same institution in 2013 in the field of nanostructured microelectrodes for electrochemical sensing. He is currently a postdoctoral researcher at the University of Toronto developing point-of-care biosensors for infectious disease detection.

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Shana Kelley was born in Philadelphia, Pennsylvania, in 1970. She is a Distinguished Professor of Chemistry, Biochemistry, Pharmaceutical Sciences, and Biomedical Engineering at the University of Toronto. Her research interests span a variety of areas including chemical biology and nanotechnology. Professor Kelley obtained her B.A. at Seton Hall University and her Ph.D. at Caltech, and she was an NIH Postdoctoral Fellow at the Scripps Research Institute.

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