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Detection of an Epstein–Barr Genome Analog at Physiological Concentrations via the Biometallization of Interdigitated Array Electrodes

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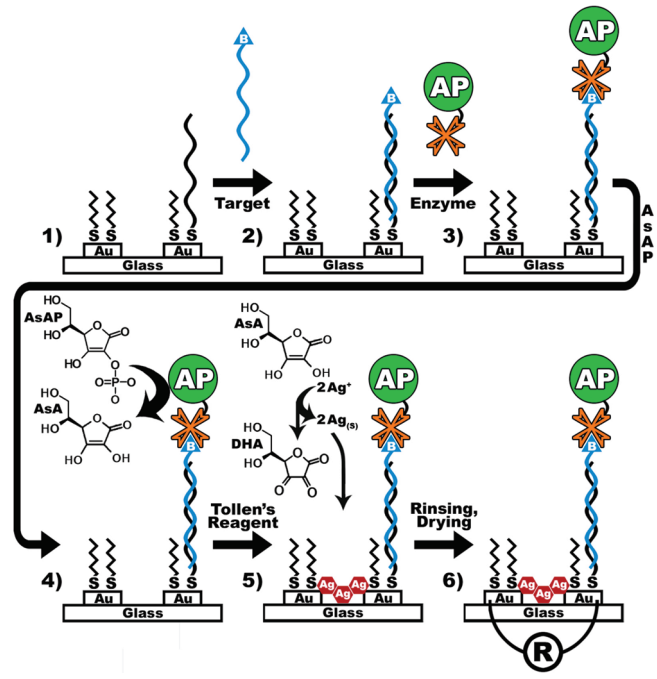
This paper reports a simple DNA sensor having a detection limit of about 24 oligonucleotides and that operates without the need for PCR amplification. The sensor platform is based on an interdigitated array (IDA) of electrodes. The electrodes are modified with DNA capture probes, which are complementary to an analog for the Epstein–Barr genome, and then exposed to an alkaline phosphatase-labeled target. The enzyme catalyzes the formation of L-ascorbic acid, which reduces Ag^+ in solution to yield conductive Ag filaments that span the gap between the electrodes of the IDA. Resistance measurements, made with an inexpensive, hand-held multimeter, signal the presence of the target. The sensor response is insensitive to the presence of a large excess of non-complementary DNA sequences.

Here we report a method for directly detecting modified, single-stranded DNA targets at concentrations normally requiring amplification by polymerase chain reaction (PCR). The time required for detection is on the order of 24 h, and the laboratory infrastructure required is minimal. The operation of the sensor is illustrated in Scheme 1. First, a DNA probe is immobilized onto the electrodes of an interdigitated array (IDA). Second, the sensor is exposed to the biotin-labeled, complementary target. Third, the hybridized target is labeled with streptavidin-conjugated alkaline phosphatase (AP). Fourth, a solution of L-ascorbic acid 2-phosphate (AsAP) is added, which is converted to L-ascorbic acid (AsA) by the enzyme. Fifth, an electrochemical reaction begins when Tollen's reagent is introduced, resulting in silver deposits that connect the electrodes of the IDA. Finally, the presence or absence of the DNA target is determined via a simple resistance measurement. Using this approach, we have been able to detect concentrations of target as low as 1 aM in the presence of a large excess of potentially interfering, non-complementary DNA.

Sensors related to the one discussed here have been described previously. For example, DNA sensors have been developed in which the binding of target oligomers leads to biometallization. This process is initiated by an enzyme that generates reducing equivalents, which in turn convert metal ions in solution to an insoluble, conductive metal (usually Ag).[1] The deposit may then be detected and quantified by stripping voltammetry.[1−4] Fritzsche and co-workers carried out biometallization between two parallel electrodes to evaluate percolation thresholds of Ag nanoparticles using atomic force microscopy (AFM).[5] Immunoassays using biometallization have also been reported using antibody-conjugated enzymes and stripping voltammetry.[2,4] In contrast to these previous studies, biometallization here is detected by measuring the resistance between parallel electrodes rather than by stripping voltammetry.

Scheme 1



Silver deposition has also been used by Fritzsche and Mirkin to amplify the presence of target DNA labeled with Au nanoparticles that catalyze Ag electroless deposition.[6−10] This approach

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can be designed so that the deposited Ag spans an insulating gap between one or more closely spaced electrodes, thereby reducing the resistance between them.[6] This is the general approach we have adapted to the device reported here.

Enzymes are sometimes used in biosensors to chemically amplify their output signal and hence lower the limit of detection.[11] In such schemes, the enzyme is used as a label that generates many detectable products tracing back to a single target. Enzymes used for this purpose are robust and have high turnover rates. One such enzyme is alkaline phosphatase (AP). Substrates for AP include 1-naphthol phosphate[12] and *p*-aminophenol phosphate (pAPP).[13] Both substrates hydrolyze rapidly in the presence of AP to yield electroactive species, but the former has poor solubility in water and the latter slowly hydrolyzes even in the absence of the enzyme.[13] Alternatively, L-ascorbic acid 2-phosphate (AsAP) is also catalytically hydrolyzed by AP to L-ascorbic acid (AsA), but it is significantly more stable in water than pAPP.[14,15] Accordingly, we have chosen to use AsAP in the present study.

Circulating nucleic acids (CNAs) are extracellular lengths of DNA or RNA found in human blood plasma and serum. They are thought to originate from abnormal apoptotic processes, necrosis, and various pathologies.[16] Healthy individuals have average serum CNA concentrations of ~36 ng/mL, but this value depends on a variety of both endogenous and exogenous conditions.[17,18] In some cases, the Epstein−Barr virus (EBV) can manifest nasopharyngeal carcinomas.[19] These carcinomas actively produce and leach copies of the viral genome into the blood at concentrations greater than 5.9 copies per microliter, approximately 10 aM, as determined by quantitative real-time PCR.[20] Using the established pathology of EBV, it is possible that carcinoma cases may be suitable as a focus for biosensor development given the relatively small signal and high CNA background. Accordingly, we have chosen a target symbolic of EBV.

In this report we present a chip-based sensor designed to directly detect single-stranded DNA. This system has a detection limit of 1 aM that circumvents PCR amplification by coupling an IDA electrode design[21] with biometallization. Detection was possible for the target at concentrations lower than those reported from EBV carcinomas, even in the presence of non-complementary DNA at concentrations corresponding to the endogenous CNA levels of healthy individuals. Following biometallization in the presence of target, the resistance of the IDA was typically lower than 5 kΩ. In contrast, negative control experiments using a noncomplementary capture probe resulted in resistance exceeding 40 MΩ. Thus, the detection signal, resistance, is at least four orders-of-magnitude lower in the presence of the target. Importantly, the only equipment required to distinguish the presence (1 aM) and absence of the target is a hand-held multimeter.

EXPERIMENTAL SECTION

Chemicals. Photoresist (AZP4620) and developer (AZ421K) were purchased from May's Chemical (Chicago, IL.) A photoresist adhesion promoter (SurPass 3000) was purchased from DisChem, Inc. (Ridgway, PA). Gold-coated glass microscope slides having a Cr adhesion layer were purchased from EMF Corporation (Ithaca, NY). Conductive glue (Silver Adhesive 503) was purchased from Electron Microscopy Sciences (Hatfield, PA). 1-Hexane thiol (97%) was obtained from Alfa Aesar (Ward Hill, MA). Silver nitrate (99.85%) and bovine serum albumin (BSA) were purchased from Arcos Organics (Morris Plains, NJ). L-Ascorbic acid 2-phosphate hydrate sesquimagnesium salt (AsAP), ammonium cerium nitrate, streptavidin-conjugated alkaline phosphatase, salmon testicular DNA, and PerfectHyb Plus hybridization buffer (henceforth referred to as hybridization buffer) were purchased from Sigma. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was obtained from Thermo Fisher Scientific. Aqueous solutions were prepared using 18 MΩ-cm water from a Millipore Milli-Q system (Bedford, MA). All chemicals were used as received.

DNA oligomers were custom synthesized by Integrated DNA Technologies (Coralville, IA). The capture DNA probe contained a 5′-thiol modification, a six-carbon spacer, a triethylene glycol spacer, and a twenty-nucleotide sequence: CAAGACAGCACTACATCCCA-3′. The target nucleotide contained a 5′ biotin modification, a 5-thymidine spacer, and the twenty nucleotide sequence TGGGATGTAGTGCTGTCTTG-3′, which corresponds to bases 72027−72047 of wild type EBV (GenBank Accession No. AJ507799).[19] Rinsing buffer was used to clean the devices and consisted of 5 mM TRIS base and 5 mM Na_2SO_4 adjusted to pH 7.4 with H_2SO_4. A noncomplementary, negative control oligomer contained a 5′-thiol modification, a six-carbon spacer, and the nucleotide sequence GCGCGAACCGTATA-3′. Similarly, a positive control oligomer contained a 5′-thiol modification, a six-carbon spacer, a triethylene glycol spacer, the twenty-nucleotide sequence CAAGACAGCACTACATCCCA, and a 3′ biotin.

Characterization. Electron micrographs were obtained using a LEO 1530 scanning electron microscope (SEM) with a GEMINI field-emission column (Carl Zeiss). Energy dispersive spectroscopy (EDS) data and maps were compiled using EDS2008 software from IXRF Systems (Houston, TX). Electrical measurements were made using a Fluke 79 Series II digital multimeter. Plasma treatment was performed in a PDC-32-G plasma cleaner (Harrick Plasma).

IDA Fabrication. Electrodes were fabricated photolithographically by patterning photoresist onto Cr-primed, Au-coated glass and then chemically etching those areas not protected by

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the photoresist. Slides were first cleaned by immersion in fresh piranha solution (3:1 mixture of concentrated H_2SO_4 to 30% H_2O_2) for 10 min and then rinsed several times with deionized water. All photolithography was performed in a low-UV light clean room. Substrates were rinsed with water and mounted on a spin coater. Adhesion promoter was applied using a syringe at a rate of 1 mL/s for 30 s onto slides spinning at 500 rpm. Following 30 s of rinsing with water, the slides were spun dry at 2000 rpm for 1 min. Sufficient photoresist to cover the surface was applied manually to the substrate. Photoresist was spread at 2000 rpm for 1 min, followed by a 2 min bake on a 90 °C hot plate and then a second bake on a 110 °C hot plate for 3 min. At this point, the substrates were mounted in a mask aligner for hard-contact UV irradiation for 7 s. The photomask pattern consisted of 100, 2 mm-long interdigitated electrodes having 10 μm (nominal) lines and spaces leading to 1.5 mm by 2 mm contact pads. After irradiation, the exposed photoresist was stripped by immersion in developer with gentle agitation for 45 s. The developed substrates were rinsed with water and dried with compressed air. Slides were prepared for chemical etching by 30 s of plasma treatment followed by 5 min on a 110 °C hot plate to remove solvent. Unwanted Au was removed from each slide by immersion in an aqueous solution containing 8% (w/v) of both KI and I_2. Slides were then rinsed and immersed in a Cr etchant containing 9% $(NH_4)_2Ce(NO_3)_6$ (w/v) in 6% $HClO_4$ (v/v).[22] Photoresist was removed by sequentially washing with acetone, absolute ethanol, water, and finally piranha. Copper lead wires were affixed to the device contact pads using Ag adhesive and secured with an insulating epoxy. Short circuits were removed by applying 40 V ac for 1 min. Finished devices were tested by measuring the resistance between electrodes, and those having resistances >40 MΩ were retained for experiments. IDAs were stored under vacuum in a desiccator until needed.

Electrode Modification. The surfaces of IDAs were modified with capture DNA and then backfilled with an alkylthiol using the following procedure. First, the device was cleaned in a plasma cleaner for 30 s, and then 40 μL of 1 μM capture DNA in 1 M HEPES buffer with 5 mM TCEP at pH 7.0 was applied to the IDA. Next, the device was incubated for 1 h at 23 ± 3 °C inside a closed Petri dish. The IDA was then gently washed with 10 mL of rinsing buffer and then dried under a gentle stream of N_2. A 40 μL drop of 1 mM 1-hexane thiol was then applied to the IDA and incubated for 1 h. Finally, the device was washed with rinsing buffer, dried under N_2, and then used immediately.

Biometallization. As illustrated in Scheme 1, the preparation and use of an IDA for target DNA detection occurs in multiple phases. Unless otherwise noted, all processing steps were carried out at 23 ± 3 °C. The modified IDA was exposed to 40 μL of biotinylated target at concentrations of 1 fM, 100 aM, 10 aM, or 1 aM and incubated for 2 h in a humidity chamber. Target DNA was prepared for use by serial dilution into either neat hybridization buffer or hybridization buffer containing 36.3 ng/mL salmon testicular DNA. After hybridization, the device was washed with 10 mL of rinsing buffer and dried with gently flowing N_2. Next, the electrode was covered with 40 μL of 0.1 mg/mL streptavidin-conjugated AP in 50 mM TRIS base, 0.5 M Na_2SO_4, 1 mM $MgSO_4$, 0.1% (v/v) Tween-20, and 1 mg/mL BSA at pH 7.4. After 15 min the electrodes were rinsed and dried. The IDA was spotted with 10 μL of 10 mM AsAP in 50 mM TRIS base, 1 mM $MgSO_4$ (pH 8.0), and returned to the humidity chamber for 1 h. Ten minutes prior to removing the device, Tollen's reagent was prepared and used without dilution.[23] Briefly, NH_4OH was added to 10% (w/v) $AgNO_3$ until the solution changed from colorless to brown, and then an additional small volume was added dropwise until the solution was colorless again. Finally, the solution was back-titrated with additional 10% $AgNO_3$ until a slight brown suspension was formed. When the AsAP incubation was complete, the electrode was placed on a 40 °C hot plate for 5 min. A 10 μL drop of Tollen's reagent was placed on the IDA, and the device was returned to the humidity chamber for an additional for 2 h. The device was rinsed with water, cleaned with gently flowing N_2, and dried overnight in air. Devices were checked for handling damage after drying. The IDA was examined for shorts visually using a microscope; the integrity of the connection between copper lead wires and their contact pads was verified using the multimeter. Finally, the multimeter was connected across the IDA leads, and the minimum stable resistance value was recorded.

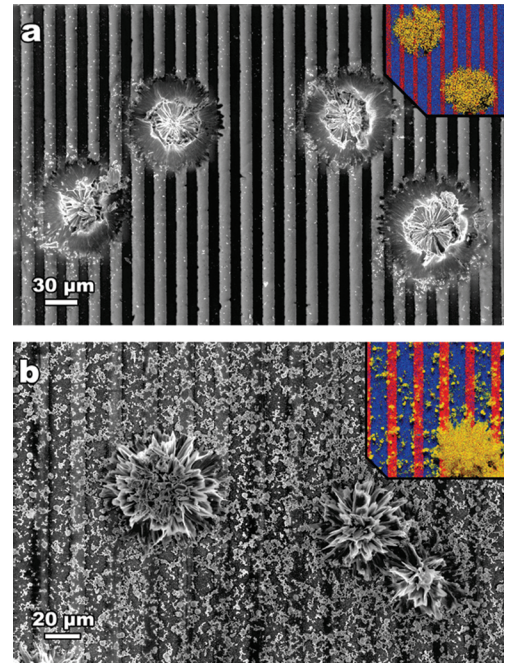

Figure 1. Scanning electron micrographs of IDAs exposed to 40 μL drops of solution containing (a) 100 aM and (b) 1 μM target DNA labeled with AP. The Au electrodes appear as dark, vertical lines, the glass interstitial regions are gray, and Ag deposits appear white. The insets are EDS maps showing the elemental composition of a portion of the micrographs. These maps indicate the locations of Ag (yellow), Au (red), and Si (blue).

RESULTS AND DISCUSSION

Fabrication and Modification of IDA Electrodes. Gold slides were patterned using photolithography and then chemically etched to yield gold features on an insulating glass substrate as shown in Figure 1. The pattern consisted of two gold contact pads

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each extending toward one pole of a 2 × 2 mm IDA. The electrodes of the IDA were positioned 10 μm apart and were connected to the multimeter using copper lead wires attached to contact pads. The electrodes were modified with a mixed monolayer of 5′ thiol-modified capture DNA and 1-hexane thiol before exposure to the target DNA solution. Hexane thiol was chosen to match the hexyl spacer present in the capture DNA and to encourage silver deposition within the interstitial glass regions of the IDA. Complete details regarding sensor fabrication are provided in the Experimental Section.

Target Detection and Silver Development. In a typical experiment, modified IDAs were exposed to a range of target DNA concentrations and then incubated for 2 h to facilitate hybridization. The target DNA was presented alone or as a "needle-in-a-haystack" of noncomplementary, potentially interfering oligomers. After hybridization, streptavidin-modified AP was bound to the surface via its affinity for the 5′ biotin on the target. Enzyme was delivered in solution with Tween-20 and BSA to prevent nonspecific adsorption. AP catalyzes the hydrolysis of the 2′ phosphate group of AsAP to yield reducing equivalents of AsA.

After incubating AsAP with the enzyme for 1 h, Tollen's reagent was added. Tollen's reagent has been used extensively as an indicator for aldehydes and in the Ag mirror reaction.[24] The solution-phase reaction between AsA and Ag^+ leads to the formation of Ag metal, which forms smooth deposits on the surface of the IDA. This two-step approach involving, first, generation of reducing equivalents and, second, addition of the Ag^+ source, is different from previously reported procedures in which the Ag^+ source is introduced at the same time as the phosphorylated precursor. In the latter case, it has been found that the enzyme is partially deactivated by Ag during the assay.[1,3] This is not a concern in the system presented here, because the enzymatic hydrolysis reaction is complete before addition of Ag^+.

We observed that the film resulting from the reduction of Tollen's reagent was more conductive than when $AgNO_3$ or Ag_2SO_4 was used as the source of Ag (data not shown). Moreover, the inclusion of a warming step prior to development enhanced the adhesion of Ag onto the glass surface between the electrodes and thus increased conductivity. An additional 2 h in the humidity chamber was found to positively affect the conductivity of the deposit.

Surface analysis by SEM (Figure 1) reveals two predominant types of features after biometallization with Tollen's reagent: dendritic Ag deposits and hemispherical clusters consisting of radiating leaflets. The metallic dendrite structures spanning the gap between electrodes are responsible for the enhanced conductivity observed in the presence of the complementary target. Dendritic growth of Ag is most apparent in Figure 1b, because this device was prepared for illustrative purposes using a very high concentration (1 μM) of enzyme-labeled target DNA. However, careful examination of the IDA shown in Figure 1a, which was prepared using 100 aM target, also reveals the presence of Ag deposits between electrodes. The insets shown in Figure 1 are element maps obtained using energy dispersive spectroscopy (EDS). These maps indicate the locations of Ag (yellow), Au (red), and Si (blue).

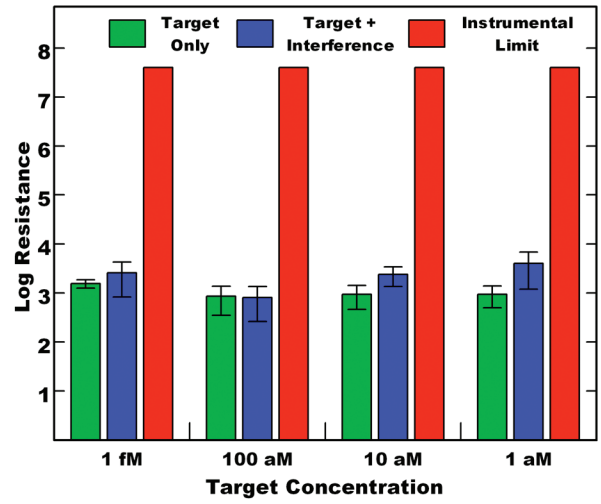

Figure 2. Resistance dose−response data for target DNA concentrations of 1 fM, 100 aM, 10 aM, and 1 aM. The green bars represent the average resistance values for devices exposed to target DNA only (non-complementary DNA was excluded). The blue bars correspond to target DNA in the presence of 36.6 ng/mL of noncomplementary, unlabeled DNA. The red bars represent the maximum threshold resistance of the multimeter (40 MΩ). Error bars represent one standard deviation from the average of three to six replicates. Prior to chemical modification, the IDA resistances of all control experiments referred to in the text exceeded 40 MΩ.

The relatively large, leafleted structures that dominate the micrographs in Figure 1 were consistently observed in target-containing assays, but they were also observed in many of the negative control experiments. Compositional analysis using EDS indicated that the clusters are mostly composed of Ag but that they also contain a small percentage of carbon. Importantly, resistance measurements carried out on negative control devices on which these features were observed showed that they do not provide an electronically conductive pathway between the interdigitated electrodes. Therefore, they do not adversely impact the sensing experiments described in the next section. The composition and morphology of these clusters is consistent with earlier studies of ascorbate-mediated Ag^+ reduction in which silver ascorbate, an intermediate species, was isolated in the form of spherical particles several micrometers in diameter.[25] By analogy to this previous study, and taking into account our finding that these features are not electronically conductive, we believe that at least the part of the clusters in contact with the interdigitated electrodes are composed of insulating silver ascorbate.

Target Dose−Response Assays. The genosensor was evaluated at concentrations of target DNA between 1 fM and 1 aM. The oligonucleotide sequence complementary to the surface-confined probe was delivered to the device alone or mixed with noncomplementary sequences, and each concentration was assayed using three to six replicate IDAs. Measured resistance values reflect the binary nature of this sensor, which yields either a positive or negative qualitative response. A positive response was defined as an IDA having a resistance <40 MΩ (the maximum threshold for the multimeter). Data for the dose−response assays, with and without non-complementary DNA, are presented in Figure 2. The key result is that resistances for devices exposed to target DNA were found to be lower than 5 kΩ whether or not

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noncomplementary, unlabeled DNA was present in large excess (36.3 ng/mL).

A series of control assays was performed to establish that the assay responds only to the labeled target. In all of the following control measurements, the IDA resistances were found to exceed 40 MΩ: (1) naked IDAs and IDAs modified with probe DNA; (2) probe-modified IDAs exposed to complementary, biotinylated DNA before and after introduction of streptavidin-modified AP; (3) naked and probe-modified IDAs exposed to Tollen's reagent. Finally, and most importantly, when an IDA was modified with 5′ thiol-modified DNA noncomplementary to the target, exposed to enzyme-labeled target DNA at concentrations spanning the range represented in Figure 2, and subjected to the conditions required for biometallization, the IDA resistance was found to be >40 MΩ.

SUMMARY AND CONCLUSIONS

The concentrations of target DNA used in these experiments are representative of EBV viral CNAs, and it is significant that such a simple sensor design can provide a qualitative genomic test without the need for PCR even in the presence of a large excess of noncomplementary oligonucleotide sequences. In the most extreme case, represented by the 1 aM test solution (Figure 2), approximately 24 copies of the target DNA were delivered to the device. This sensor is sufficiently easy to use and requires such minimal instrumentation that one can imagine it being implemented in remote, point-of-care applications where a simple "yes" or "no" response is required.

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