Immunoassays for Proteins

DNA-Based Amplified Bioelectronic Detection and Coding of Proteins**

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As research moves into the area of proteomics, scientists are faced with the challenge of developing effective tools for identifying, quantitating, and characterizing proteins.[1,2] Such new methods for analyzing proteins have the potential to improve drug discovery as well as the diagnosis and understanding of various disease states. The transduction of protein recognition events is of considerable interest for meeting this goal. Most clinical diagnostic methods for detecting proteins are based on conventional enzyme immunoassays.[3,4] These and other antibody-based techniques hold great promise for designing microarrays for detecting multiple protein targets.[5,6]

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Here we report on a new and powerful bioelectronic protocol for the amplified electrical detection and coding of proteins. Electronic transduction of protein interactions is a major challenge in protein-based bioelectronics, while electrical devices are ideally suited for meeting the size, low-cost, and power requirements of point-of-care protein testing. Ultrasensitive electrical immunoassays have been developed by using enzyme labels as well as inexpensive and compact instrumentation.[7,8] In a recent study Nam et al.[9] demonstrated a highly sensitive DNA-based optical (scanometric) method for the detection of proteins. While DNA can act as an ideal molecular label,[10] its utility in electrochemical detection has not been documented. Our new amplified bioelectronic protein detection takes advantage of the state-of-the-art in electrical DNA detection methods,[11] including the electroactivity and highly sensitive stripping response of the guanine (G) and adenine (A) nucleobases,[11,12] the amplification potential of polymeric beads carrying numerous DNA tags, and the ability to create distinct oligonucleotide-identifiable electrical bar codes. Electrical measurements based on the redox activity of purine nucleobases have been widely used for label-free DNA hybridization assays.[13–15]

The bioelectronic protocol (Figure 1) involves a sandwich immunoassay based on two antibodies linked to magnetic

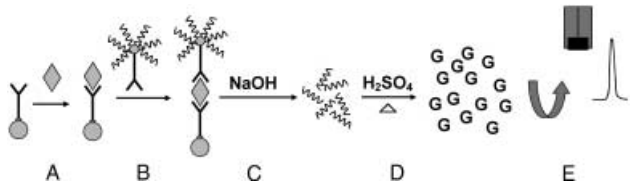

Figure 1. Schematic representation of the analytical protocol: A) binding of the IgG analyte to the anti-IgG-coated magnetic beads; B) secondary binding and capture of the DNA/anti-IgG-functionalized polystyrene tags to the magnetic beads coated with the antibody–antigen complex; C) release of the DNA marker using 0.05 M NaOH; D) acid dipurinization; E) electrochemical (adsorptive chronopotentiometry) detection of the acid-released purine bases with a pyrolytic graphite electrode. Magnetic separation is used after steps A and B to remove unwanted constituents and unbound tagged spheres, respectively.

beads and DNA-functionalized polystyrene (PS) spheres (steps A and B), followed by alkaline release of the oligonucleotide strands from the beads (C), the acidic dipurinization of the released DNA (D), and adsorptive chronopotentiometric stripping measurements of the free nucleobases at a pyrolytic graphite electrode transducer (E). The last step involves adsorptive accumulation of the purine bases followed by passage of a constant anodic current through the electrode.[12]

The protein-recognition event leads to a three-dimensional protein-linked particle assembly (Figure 2a), with the 1.5-μm anti-IgG-coated magnetic beads (dark) cross-linked to the 0.5-μm DNA-loaded PS spheres (bright) and with the antibody–antigen–antibody complex acting as "glue". Similar biorecognition-induced particle aggregations have been reported in nanoparticle-based electrical and optical DNA hybridization assays.[16] No such cross-linking was observed in

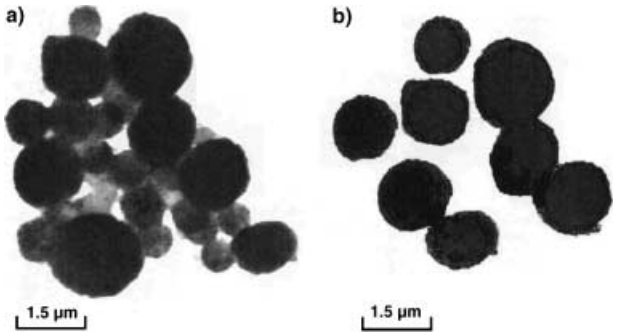

Figure 2. a) TEM image of the protein-linked particle assembly produced following a 30 min incubation in the 10 ng mL^{-1} solution of IgG, b) same as (a) but without the IgG target. The images were taken with a Hitachi H7000 instrument, operated at 75 kV, after washing the particle–protein assembly with autoclaved water, and placing a 5-μL droplet of the particle aggregate onto a carbon-coated copper grid (3-mm diameter, 200 mesh) and allowing it to dry.

a control experiment performed in the absence of the target protein (Figure 2b). Apparently, the DNA/anti-IgG-functionalized (IgG = immunoglobulin G) PS spheres are effectively removed by magnetic separation, thus leaving the dark magnetic beads behind.

The quantitative assessment of the hybridization-free DNA-based bioelectronic protein assay that does not utilize the polymerase chain reaction (PCR) is based on monitoring the dependence of the purine (marker) oxidation peak arising from the immunological reaction. Figure 3a displays typical chronopotentiograms for increasing levels of the IgG target protein (1–100 ng mL^{-1}; I–III). Well-defined guanine signals are observed for these low concentrations of protein after

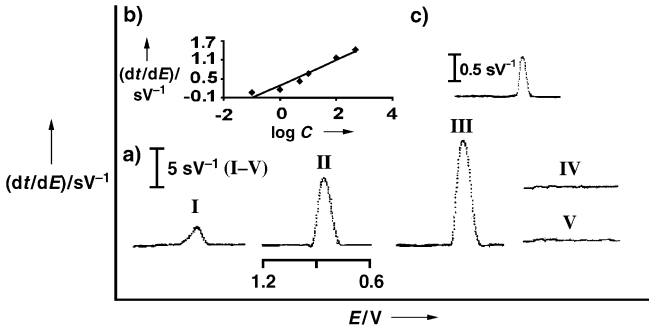

Figure 3. Stripping potentiograms for increasing levels of the target IgG(a): I) 1 ng mL^{-1}; II) 10 ng mL^{-1}; III) 100 ng mL^{-1}; IV) 0 ng mL^{-1}; and V) response to 1 mg mL^{-1} bovine serum albumin. Also shown are the resulting calibration plot over a range of 0.1 to 500 ng mL^{-1} (b) and a stripping potentiogram for a 0.01 ng mL^{-1} solution of IgG (c). Amounts of magnetic beads and functionalized PS spheres: 25 μg and 5 mg, respectively; incubation time (of each recognition event): 30 min. The DNA/anti-IgG-functionalized PS spheres were prepared by gently mixing 5 mg of the particles in 100 μL PBS solution containing 56 μg mL^{-1} dG_{25} and 10 μg mL^{-1} anti-IgG for 30 min. The protein-linked particle assembly was dispersed in 50 μL 0.05 M NaOH to release the DNA marker; 10 μL 3 M H_2SO_4 were then added and the solution was heated to dryness. An acetate buffer solution (1 mL, 0.5 M, pH 5.9) was used to transfer the digested DNA into the detection cell. Electrode preconditioning: 1 min at 1.25 V; accumulation: 2 min at −0.10 V; stripping current: 5 μA.

incubation for 30 minutes. The corresponding calibration plot of response versus log[protein] (Figure 3b) is linear over the 0.1–500 ng mL^{-1} range and is suitable for quantitative work. A similar logarithmic dependence was reported for other particle-based bioassays[16a,17] and was attributed to changes in the degree of aggregation and blocking of binding sites at high ligand concentrations.[18] The coupling of carrier-sphere amplifiers with the preconcentration feature of electrochemical stripping detection leads to extremely low detection limits. The response obtained with the 10 pg mL^{-1} DNA target (Figure 3c) indicates a detection limit of around 2 pg mL^{-1} (13 fM), that is, 0.65 amol or 4×10^5 protein molecules in a 50 μL sample. Such a low detection limit compares favorably with values obtained with common immunological assays such as the enzyme-linked immunosorbent assay (ELISA).[17] Further extension of the detection limits—to the attomolar level–-could be achieved (at the cost of higher procedural complexity) by replicating the DNA tags by PCR, in a manner analogous to immuno-PCR optical tests.[9,10] The use of longer oligonucleotide strands and/or the electrocatalytic action of a $[Ru(bpy)_3]^{2+}$ redox mediator should also be useful for obtaining further amplification. The high sensitivity is coupled with excellent selectivity and the absence of nonspecific binding effects. No background signals are observed in control experiments without the target protein or when using a huge (ca. 10^3) excess of bovine serum albumin (Figure 3a, IV and V, respectively). Such behavior reflects the shielding of the magnetic beads and the efficient removal of unwanted constituents (including unbound tagged spheres) by magnetic effects. This finding is encouraging, as proteins tend to exhibit greater nonspecific binding to solid supports than do short oligonucleotides. The sensitive and specific response is coupled with high reproducibility. The precision was estimated from a series of six measurements of samples containing 10 ng mL^{-1} of the target protein which yielded a mean peak area of 432 ms and a relative standard deviation of 5%.

Since the amplified detection of protein interactions relies on the use of numerous oligonucleotide tags per binding event, proper attention must be given to the surface coverage of the tagged polymeric spheres. A coverage of around 7.5×10^4 dG_{25} oligonucleotides per PS sphere (that is, the binding event) was estimated from a separate electrochemical experiment in which the guanine response of a given amount of the DNA-loaded spheres was compared with that of a standard solution of free guanine. Such a loading corresponds to an average surface coverage of 9.95×10^{12} oligonucleotide strands per cm^2 (if each sphere is assumed to have an area of 0.75 μm^2). This coverage approaches the high surface densities (3×10^{13} cm^{-2}) common to self-assembly of thiolated DNA on gold electrodes.[19] Such optimal surface coverage was obtained by incubating 5 mg of the PS beads in a 56 μg mL^{-1} solution of dG_{25} for 30 minutes. This concentration of DNA was selected by monitoring the guanine response over a wide concentration range of dG_{25}. The guanine signal increased rapidly upon increasing the concentration of dG_{25} (in the "loading" solution) from 10 to 55 μg mL^{-1} and almost leveled off thereafter. The optimal anti-IgG loading on the PS spheres was obtained using a 10 μg mL^{-1} solution of the antibody (containing 56 μg mL^{-1} dG_{25}). Other factors influencing the response were assessed. The highest sensitivity (along with elimination of nonspecific binding effects) was obtained by simultaneous loading of the dG_{25} and anti-IgG, 25 μg of the IgG-coated magnetic beads, 5 mg of the DNA/anti-IgG-functionalized PS spheres, and using an accumulation potential of −0.1 V over 2 minutes (see Supporting Information for details and related data). We also evaluated several carbon-electrode transducers, including carbon paste, graphite pencil, carbon-nanotube-coated glassy carbon, and pyrolytic graphite, and found that the last of these offered the most favorable purine response in connection with a preconditioning for one minute at +1.25 V.

In addition to single-analyte formats, the new DNA-based bioelectronic protocol offers great promise for the electrical detection of multiple proteins. For this purpose, it is possible to create distinct identifiable oligonucleotide barcodes for electrochemical immunoassays. In particular, a large number of recognizable electrochemical signatures can be obtained by designing oligomers with different predetermined A/G ratios. Initial assessment of this electrical coding strategy appears to be very promising. For example, Figure 4 displays the chronopotentiometric immunoassay response obtained with different oligonucleotide labels. As expected, the dG_{25} and dA_{25} tags yield well-defined chronopotentiometric peaks at 0.83 (Figure 4a) and 1.05 V (Figure 4b), respectively, while the $dG_{15}A_{10}$ tracer leads to two (G and A) peaks at similar potentials (Figure 4c). The higher sensitivity of the adenine nucleobase should be taken into account when designing

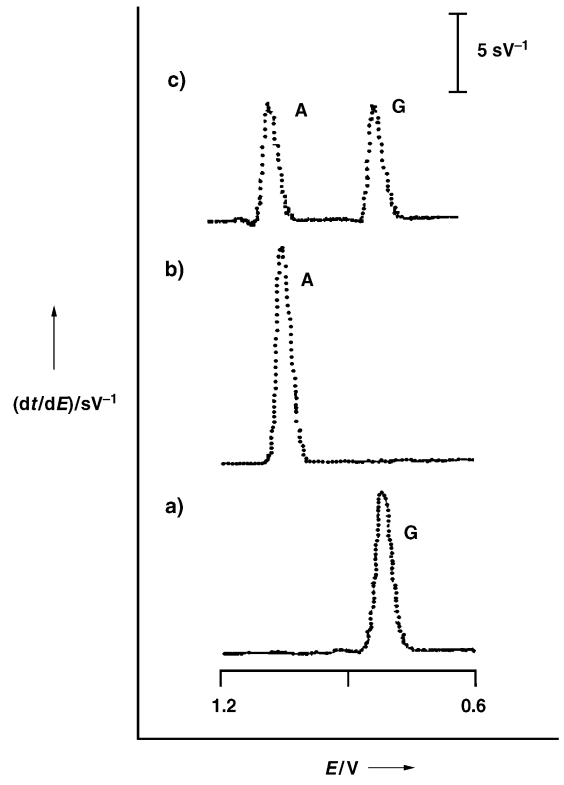

Figure 4. Chronopotentiometric immunoassay signals for a 100 ng mL^{-1} solution of IgG using different DNA markers: a) dG_{25}; b) dA_{25}; c) $dG_{15}A_{10}$. Other conditions, as in Figure 2.

biological barcode patterns. A wide range of distinguishable signal intensities (namely, A/G ratios) are currently being considered for increasing the number of uniquely identifiable electrical barcodes and hence proteins.

In summary, we have demonstrated a new bioelectronic strategy for ultrasensitive measurements of proteins based on the use of nucleic acid tracers. The resulting electrical detection scheme incorporates the high sensitivity, selectivity, and miniaturization advantages of electrical assays. The remarkable sensitivity reflects the use of numerous oligonucleotide tags per protein-binding event and the amplified detection of protein interactions has been coupled to an efficient magnetic removal of unwanted constituents. We are currently designing a wide range of oligomers with different predetermined sequences for multiple protein analysis. The DNA-based electrochemical technology is thus expected to open new opportunities for protein diagnostics, microarrays, and microchips, as well as for bioanalysis in general.

Experimental Section

Chronopotentiometric measurements were performed with a computer-controlled potentiometric stripping unit PSU20 (Radiometer) using TAP2 software (Radiometer). The immunological binding reactions were performed on a MCB 1200 Biomagnetic Processing Platform (Sigris Research, Fremont, CA, USA). A Micromax centrifuge (Thermo IEC, MA) was used for removing the excess reagent during the preparation of the polystyrene bead tags. The detection was carried out in a 1.5-mL electrochemical cell containing a three-electrode system (a pyrolytic graphite (Advanced Ceramics, Cleveland, OH) disk working electrode (geometric area 0.16 cm^2), an Ag/AgCl reference electrode, and a platinum wire counter-electrode). The pyrolytic graphite electrode was initially abraded using 600-grit silicon carbide paper, followed by thorough washing with deionized water and drying under nitrogen. The protein-linked particle assembly was characterized by using a Hitachi H-7000 transmission electron microscope.

Anti-mouse IgG–biotin conjugate, mouse IgG, sodium phosphate (NaH_2PO_4), NaOH, NaCl, and sulfuric acid were purchased from Sigma. Tween 20 was purchased from Aldrich. The anti-IgG-coated magnetic beads (1.5 μm) and the streptavidin-modified polystyrene beads (0.49 μm) were obtained from Bangs Laboratories (Fishers, IN, USA). Oligonucleotides with 5′-biotin modification were received from Life Technologies (Grand Island, NY, USA). The sequences of the oligonucleotides are as follows:
5′-biotinylated-GGGGGGGGGGGGGGGGGGGGGGG;
5′-biotinylated-AAAAAAAAAAAAAAAAAAAAAAAA;
5′-biotinylated-GGAGGAGAAGGAGGAGGAGGAGAGA.
All the other reagents were analytical grade and were prepared using nanopure water (specific resistance 18 ohm cm^{-1}) and autoclaved water.

The DNA/anti-IgG-functionalized polystyrene microspheres were prepared daily by adding the nucleic acid tracer and anti-IgG (final concentrations: 56 and 10 μg mL^{-1}, respectively) into 1.5-mL vials containing streptavidin-coated polymeric microspheres (5 mg, initially washed twice with phosphate-buffered saline (PBS) and separated by centrifugation at 13000 rpm for 3 min) in PBS solution (50 μL); the required amount of PBS buffer was added to obtain a final volume of 100 μL. This was followed by incubation for 30 min at room temperature with gentle mixing. The beads were then washed and separated as above with PBST buffer (100 μL, 0.1 M PBS at pH 7.4 containing 0.1 % Tween 20) and the resulting pellet of anti-IgG/DNA-loaded microspheres was suspended in PBS buffer (25 μL).

The bioelectronic assays involved transferring 25 μg of the anti-IgG coated magnetic beads into 1.5-mL centrifuge vials, washing them twice with PBS buffer (100 μL), and suspending them in PBS buffer (50 μL) solution containing the target protein. The immunological reaction proceeded for 30 min at room temperature with gentle mixing. After a magnetic separation, the magnetic beads coated with the antibody–antigen complex were washed twice with PBST buffer (100 μL) and suspended in PBS buffer (25 μL). This volume of the immunocomplex-captured magnetic-bead solution was then mixed with the DNA/anti-IgG-functionalized PS microsphere solution (25 μL), and incubated for 30 min with gentle shaking at room temperature. A magnetic separation and multiple washing with PBST buffer (100 μL) were then carried out. The beads were then dispersed in a solution of NaOH (0.05 M, 50 μL) for 10 min with gentle shaking to release the DNA marker. The supernatant was transferred to a 1.5-mL glass cell, followed by addition of 3 M H_2SO_4 (10 μL). The acid dipurinization proceeded by heating to dryness. An acetate buffer solution (1 mL, 0.5 M, pH 5.9) was used to transfer the digested DNA into the electrochemical cell.

Chronopotentiometric stripping measurements of the released purine nucleobases were performed at a pyrolytic graphite electrode following 1 min preconditioning at 1.25 V, using a 2 min accumulation at −0.1 V in a stirred acetate buffer solution (0.5 M, pH 5.9; 1 mL). Subsequent stripping was carried out after a 10 s rest period (without stirring) using an anodic current of +5.0 μA. The stripping data were filtered and baseline corrected using the TAP2 software.

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