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Multiplexed Detection of Protein Cancer Markers with Biobarcoded Nanoparticle Probes

Savka I. Stoeva,[†] Jae-Seung Lee,[†] Jennifer E. Smith,[†] Steven T. Rosen,[‡] and Chad A. Mirkin*,[†]

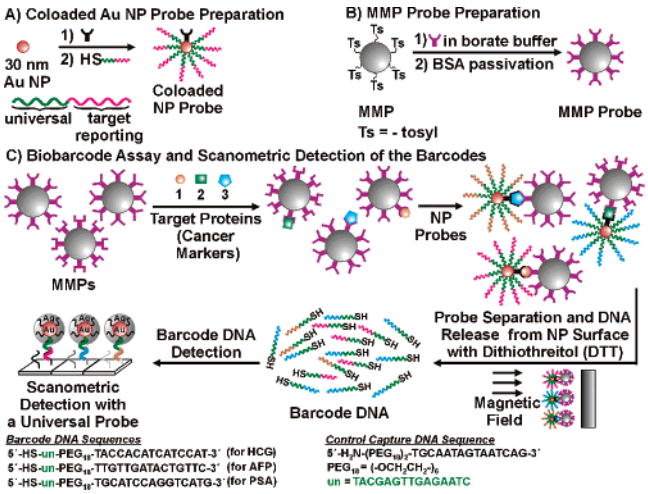
Department of Chemistry and International Institute for Nanotechnology, Northwestern University, Evanston, Illinois 60208, and Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, Illinois 60611

Received February 23, 2006; E-mail: chadnano@northwestern.edu

Highly sensitive and selective immunoassays capable of detecting proteins in a multiplexed fashion are essential for disease diagnostics, drug screening, and biodefense applications.[1] As our understanding of disease increases, multiplexed protein assays will become increasingly important. For many types of cancers, it will not be sufficient to study a single protein marker, but rather a suite of markers that allow one to diagnose and follow the disease as a function of treatment.[1−3] Heterogeneous enzyme-linked immunosorbent assays (ELISAs) coupled with optical probes (chemiluminescent or fluorophore) (1) lack the required sensitivity to diagnose and study many types of diseases, including many forms of cancer, (2) provide slow antibody−antigen binding kinetics, leading to long assay times at low target levels, and (3) in the case of multiplexed analysis, suffer from overlapping spectral features, nonuniform photobleaching rates, false positive signals, the need for multiple laser excitation sources, and complex instrumentation for assay readout.[4] The sensitivity of ELISA is significantly improved by the immuno-PCR method, whose major drawbacks are the necessity of cumbersome antibody−DNA conjugation protocols and PCR requirements, which ultimately have hampered its widespread use for multiplexed protein analysis.[5] To circumvent the aforementioned limitations, other alternative approaches for labeling and detection of biomolecules have been suggested, such as DNA-directed immobilization of proteins on supports,[6] fluorophore-encoded microspheres,[7] nanowires,[8] microcantilevers,[9] electrochemical coding,[10] multimetal microrods,[11] and metal and semiconductor nanoparticle probes.[12]

Oligonucleotide-functionalized gold nanoparticles (Au NPs) have a demonstrated advantage over conventional probes in a variety of biodetection schemes as a result of their unique chemical and physical properties.[13] The biobarcode assay, based on Au NPs functionalized both with oligonucleotides (the barcodes as a surrogate target) and a target recognition element (an antibody for proteins and a portion of the barcode for nucleic acids), is a powerful amplification and detection system for proteins[14] and nucleic acids.[15] It allows one to rapidly detect nucleic acid targets with sensitivity of PCR and protein targets at limits of detection that are 4−6 orders of magnitude lower than ELISAs, depending upon capture antibody and background signal. In the research and clinical diagnostic arenas, the biobarcode assay offers the following opportunities: (1) the potential use of new protein markers that could not be utilized for disease diagnosis with current technology because of sensitivity limitations;[14b] (2) the assessment and study of disease recurrence using existing markers; and (3) the availability of a large library of discernible barcode oligonucleotides allowing the simultaneous detection of a panel of markers with high selectivity enabling an accurate diagnosis of a complex disease. Until now, however, it has remained unclear whether the biobarcode assay could be utilized for the multiplexed detection of proteins. The realization of a multiplexing system faces significant challenges, including the elimination of probe set/target set cross reactivity, selection of well-characterized high-affinity antibodies, minimization of nonspecific binding, synthesis of stable probes, and design of noncrossreacting reporters.

Scheme 1. Biobarcode Assay for Multiplexed Protein Detection



Herein, we present the first example of multiplexed protein detection with the biobarcode assay in the context of three established cancer markers at low-femtomolar concentration: prostate specific antigen (PSA, prostate cancer marker), human chorionic gonadotropin (HCG, testicular cancer marker), and α-fetoprotein (AFP, hepatocellular carcinoma marker).[3] In addition, HCG and AFP in combination are suggested as the most effective markers for the diagnosis and monitoring of patients with testicular germ-cell tumors.[3] The assay can be performed in a 96-well plate format in a high-throughput manner in buffer or serum.

The multiplexed barcode assay utilizes three 30 nm Au NP probes, each cofunctionalized with an antibody (monoclonal or polyclonal) that recognizes the target protein and barcode oligonucleotides as surrogates for the target antigen (Scheme 1A). Note that half of the barcode for each target is the target-reporting oligonucleotide probe, while the other half is identical for all the barcodes and is in effect a universal sequence (Scheme 1A). Such a barcode design enables one to use only one type of 13 nm Au NP probe for the scanometric identification of the barcodes (Scheme 1), which significantly simplifies the system and reduces assay time and effort. The target antigens are captured in solution by three magnetic microparticle (MMP) probes, each conjugated with a monoclonal antibody specific for an epitope of the target antigen different from the one recognized by the Au NP probe. The target-MMP complexes are then sandwiched by a Au NP probe with an antibody that can bind to the target in a different region than the MMP (Scheme 1B,C). These complexes are isolated with a magnetic field and washed, and the barcode strands are released by a ligand exchange process induced by the addition of dithiothreitol (DTT, Pierce).[15b] The barcode strands are then identified by the chip-based

[†] Department of Chemistry and International Institute for Nanotechnology.
[‡] Robert H. Lurie Comprehensive Cancer Center.

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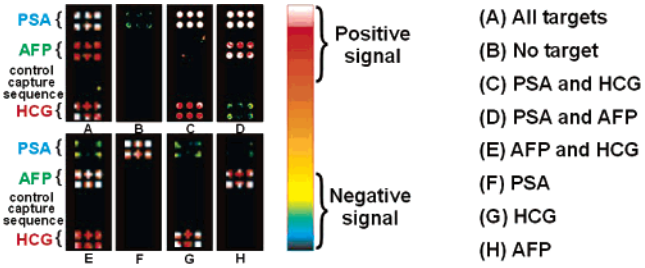

Figure 1. Scanometric detection of the barcodes. The gray scale images from the Verigene ID system are converted into a color scale using GenePix Pro 6 software (Molecular Devices).

scanometric method,[13b] developed in our laboratory and commercialized by Nanosphere, Inc.

Each Au NP probe (30 nm diameter, Ted Pella, Inc.) is cofunctionalized with a detection antibody and barcode DNA strands (~180 barcodes/particle),[16] following a new chemical procedure (Supporting Information). The Au NP probes are stored at a concentration of 6 nM, with the excess barcode DNA removed in 0.15 M NaCl, 0.025% Tween 20, 0.1% BSA, and 10 mM phosphate buffer, pH = 7.2 (assay buffer). The NP probe solution, composed of all three NP probes (NP multiplexing solution), is prepared by diluting equal volumes of each NP probe (6 nM) in assay buffer to a final NP concentration of 240 pM in the presence of 6.6 mg/mL tRNA to reduce nonspecific binding (Sigma-Aldrich).[14d] Each MMP probe is prepared by coupling tosyl-functionalized MMPs (1 μm diameter, Invitrogen) to the primary amino groups of the corresponding antibodies in borate buffer at pH = 9.5 at 37 °C. The surfaces of the MMP probes are passivated with bovine serum albumin (BSA, R&D Systems). The MMP probes are stored in assay buffer at a concentration of 10 mg/mL. The MMP probe solution, composed of all three MMP probes (MMP multiplexing solution), is prepared by diluting equal volumes of each MMP probe (10 mg/mL) in assay buffer to a final total MMP concentration of 0.0125 mg/mL.

To evaluate the selectivity of the system, eight tests are run with different combinations of the target antigens (Figure 1). In the first combination, all three targets are present. In the next seven tests, we systematically combine targets to evaluate the multiplexing capability of the barcode method. Significantly, the operator does not know the contents of each sample. The concentrations of the target antigens are kept constant for all experiments (each 170 fM final concentration in the assay). First, the antigens are captured by the MMP probes at 25 °C for 1.5 h, the MMP/target complexes are washed, and the NP probes are allowed to bind at 25 °C for 1 h. Next, the sandwiched structures are washed seven times with assay buffer, and the barcode DNA strands are released by the addition of 0.5 M DTT in 0.5 M NaCl, 0.01% Tween 20, 10 mM phosphate buffer, pH = 7.2 (scanometric buffer). The barcodes are detected with the chip-based scanometric method, and the signal is identified with a Verigene ID system (Nanosphere, Inc.) that measures the scattered light from the developed spots after silver amplification.

The scanometric identification of the barcode oligonucleotides is presented in Figure 1. In each combination, the presence of target proteins is accurately correlated to a high-intensity positive signal (white/red color), clearly distinguishable from the low intensity background negative signal (yellow/black color). The quantitation of the net signal intensities is given in the Supporting Information (SI, Figure 1S). The difference in the signal intensities of the positive signals are likely related to the different binding constants of the antigens with the corresponding antibodies.[17] No hybridization is observed between the released barcode strands or the universal probe and the negative control capture DNA sequence printed on the chip. Significantly, one also can utilize the developed multiplexed barcode assay in a complex medium, such as goat serum, and selectively detect the three antigens at low-pM concentration in an undiluted serum or 170 fM in diluted serum (1:200, see SI).

In conclusion, we have demonstrated the highly selective multiplexed detection of three protein cancer markers in buffer and serum media utilizing a new multiplexed version of the biobarcode amplification method. The results presented here validate the multiplexing capabilities of the barcode method and open new avenues for developing highly selective panel assays for the early detection of a wide range of diseases and potentially the evaluation of residual disease and disease recurrence, where high sensitivity is essential for early identification. In addition to the uses of the high sensitivity of the barcode assay and these markers to diagnose cancer, two of these markers, AFP and HCG, are important for screening fetal Down's syndrome. Since AFP is at a low concentration in Down's syndrome pregnancies, high sensitivity and multiplexed panels could contribute to an earlier diagnosis of this fetal abnormality.[18]

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Supporting Information Available: Detailed procedures for Au and MMP probes preparation, multiplexed barcode assay in buffer and goat serum, and scanometric barcode DNA detection. This material is available free of charge via the Internet at http://pubs.acs.org.

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