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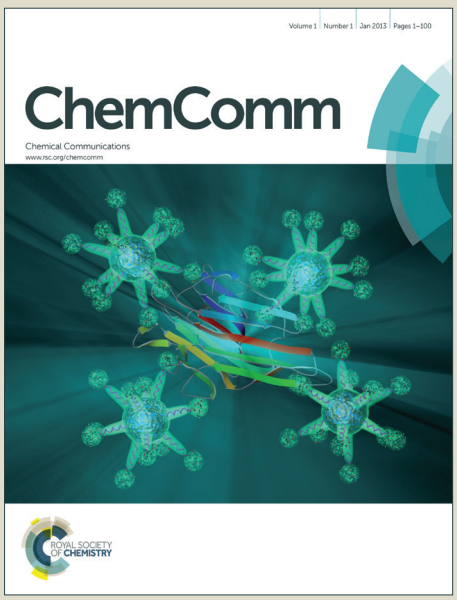

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ARTICLE TYPE

A scanometric antibody probe for facile and sensitive immunoassays

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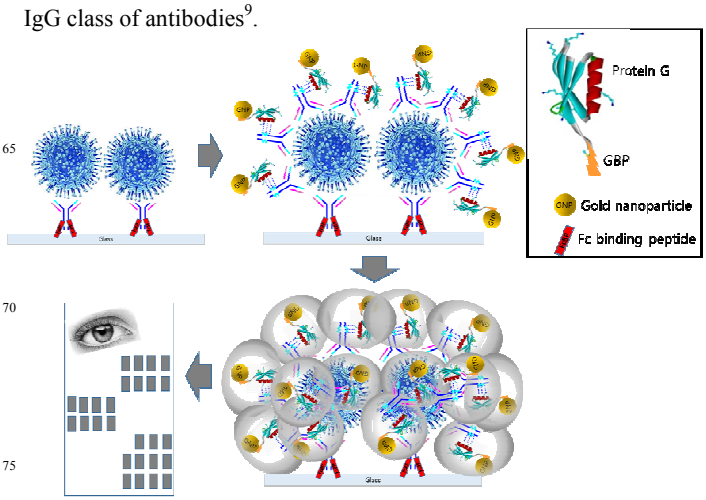
We have developed a novel scanometric antibody probe for rapid, sensitive, and naked-eye-visible immunoassays. Using the probe, we clearly demonstrated successful scanometric detection and identification of influenza A viruses on a microarray. In addition, the sensitivity of the scanometric immunoassay was comparable to that of the fluorescence-based method.

Immunoassays are commonly used in clinical diagnostic, pharmaceutical, and bio-analytical applications[1]. Immunoassays are widely used for the analysis of clinical samples in the picogram to nanogram range. The signal generated by the label attached to either the antibody or the antigen indicates the specific interaction between the antibody and antigen; therefore, proper labels and the availability of an antibody with sufficiently high affinity for and specificity to a small amount of antigen are crucial for the high sensitivity of an immunoassay[2].

Molecular labels, which contribute to the high sensitivity of immunoassays, have shifted from radioisotope-based labels to non-isotopic labels because of the safety concerns associated with radiation, the short self-life of radioactive labels, and the requirement for a radiation facility. The shift has occurred despite their many advantages over non-radioactive labels, including high sensitivity, high resolution, and quantifiable results. In efforts to overcome the health-hazard issues associated with radiation, fluorescent and enzymatic tags conjugated to the antibodies have become the most commonly used labels for the detection of antigens. However, enzymatic and fluorescent tags require antibody modifications or complicated chemical conjugations of the antibody to the tags, which may lead to defective antibody function and introduce a quantitative bias, resulting in a less sensitive assay. Fluorescent probes often produce inconsistent signals due to photo-bleaching and require dedicated and expensive analytical equipment, which prevents fluorescence-based immunoassays from being conducted in point-of-care applications[3]. Inorganic quantum dots with improved photostability against photo-bleaching and superior optical properties compared with organic fluorescent dyes have been developed for label-based detection[4].

Worldwide, influenza viruses result in substantial economic loss and are recognized as major threats to public health. Rapid virus diagnosis can aid in the control of large virus outbreaks and help to reduce drug abuse, which leads to the generation of antibiotic-resistant viruses[5]. Commercially available immunoassay test kits, such as Directigen[6] or Binax[7], provide rapid test results, but they have poor sensitivity and are expensive[8]. Poor sensitivity in immunoassays leads to use of inappropriate medication; thus, there remains a need for a rapid and sensitive diagnostic test to identify influenza virus. An ideal immunoassay at the point of care should not require expensive instrumentation for the detection and must be fast, simple, and cost-effective.

Protein G from streptococcus specifically binds to the Fc domain of an antibody without any chemical modification for its binding. Because protein G binds a broad range of mouse and human IgG subclasses, it is now commonly used for purifying the IgG class of antibodies[9].



Scheme 1 Schematic illustration of naked-eye analysis of an immunoassay using a scanometric protein G-GBP universal antibody probe.

In the present study, we developed a novel scanometric antibody probe as a universal antibody signal enhancer for immunoassays. This probe allows for an extremely simple, rapid, and efficient immunoassay because it can be applied directly to the primary antibody without a further incubation step with a secondary antibody, any subsequent complicated chemical steps, or an enzymatic reaction for generating signals as in conventional immunoassays. More importantly, this probe enables analysis of the immunoassay with the naked eye or with an optical scanner. We present a highly sensitive immunoassay for the detection of influenza viruses using this scanometric antibody probe and clearly demonstrate that the sensitivity of the scanometric

immunoassay using this probe is comparable to that of the fluorescence-based method.

The "naked-eye analysis" immunoassay strategy is illustrated in Scheme. 1. The novel immunoassay probe contains protein G fused with gold-binding peptide (GBP). Combinatorially selected GBPs have been widely employed on gold surfaces or gold nanoparticles as linkers for protein immobilization and for the development of biosensing devices[10]. The protein G-GBP probe is first labeled with gold nanoparticles, and then the protein G part of the gold-labeled probe is attached to the Fc domain of the antibody, followed by silver enhancement of the gold nanoparticles, permitting naked-eye detection of the probe without incubation with a labeled secondary antibody as in conventional immunoassays[11]. Upon attachment of the probe, the primary antibody retains the optimal conformation for its interaction with the corresponding antigen and thus offers a significantly more sensitive assay compared with a randomly labeled antibody with a fluorescent dye or enzyme. More importantly, this probe can be applied to any IgG antibody, thus working as a universal signal-enhancing immunoprobe. To the best of our knowledge, this is the first report using a GBP as part of a detecting probe rather than as a linker for immobilization on a gold surface.

A codon-optimized construct of a GBP (MHGKQATSGTIQS)[12] N-terminally fused to protein G and His-tagged at its C terminus was synthesized, cloned into a pET21a vector, expressed in *E. coli* strain BL21 with induction by IPTG, and purified with Ni-NTA resin. The protein G-GBP fusion protein was detected at its predicted size of 25 kDa by SDS PAGE analysis (Fig. S1a). The expressed protein G-GBP fusion protein (0.1 mg/ml) was treated with gold nanoparticles (Au) in PBS (20 nM) at 4 °C for 16 h, and unreacted GBP fusion protein was removed via centrifugation. A protein G-GBP linked layer on Au appeared as a shell around the Au with the thickness of 6.7±1.1 nm, as shown in TEM images (Fig. S1b). In the UV-visible spectra, the Au adsorption peak at 520 nm was shifted to 526 nm upon protein-GBP shell formation (Fig. S1c). The gold nanoparticle-attached protein G-GBP probe (protein G-GBP-Au) was incubated with an antibody (0.1 mg/ml, human IgG) at room temperature for 30 min, and unreacted antibody was removed via centrifugation. The protein G-GBP-Au-antibody complex was heated at 95 °C for 5 min and analyzed by SDS-PAGE and western blotting. The molecular weight of the antibody expected was 50 kDa for the heavy chain and 25 kDa for the light chain. Fig. S2 shows two bands at approximately 25 kDa and 50 kDa, demonstrating that a binding interaction occurred between the protein G-GBP-Au probe and the antibody.

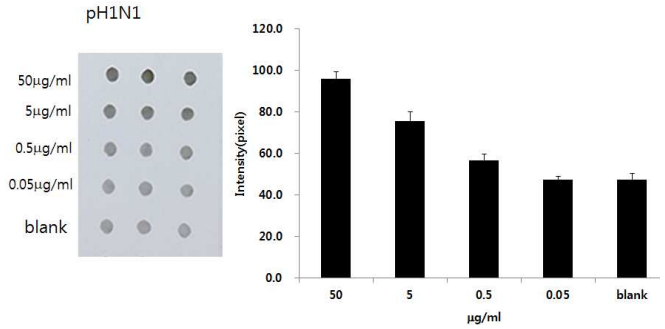


Fig. 1 Graph of the 8-bit grayscale values and antibody array following the enhancement process for various concentrations of the A/california/04/2009 (pH1N1) virus target (each probe was spotted in triplicate).

The concentration-dependent signal enhancement of the protein G-GBP-Au probe was explored on a glass surface (Fig. 1). A carboxylated glass surface was activated with NHS for the amine conjugation of Fc binding peptide (DCAWHLGELVWCT, 100 μM). A monoclonal antibody against influenza A/california/04/2009 (H1N1) virus was applied to the Fc binding peptide covered on the glass surface, followed by treatment with various concentrations of A/california/04/2009 (H1N1) virus ranging from 0.05 μg/ml to 50 μg/ml. The surface was subsequently treated with polyclonal antibodies against A/california/04/2009 (pH1N1) virus, and the protein G-GBP-Au probe was applied, followed by silver enhancement of the probe. After optical scanning, a positive correlation between the intensity of the gray levels and the concentration of the virus could be observed with the naked eye. We estimated the detection limit of H1N1 using the protein G-GBP-Au probe to be 0.16 μg/ml.

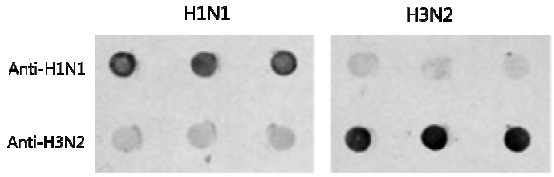


Fig. 2 Analysis of influenza virus A subtypes (H1N1 and H3N2) on an antibody chip by an optical scanner image. Hybridized pH1N1 or H3N2 virus was detected with a signal enhancement of the protein G-GBP-Au probe-bound antibody specific for the influenza virus nucleoprotein. (0.1 mg/ml).

We next applied the protein G-GBP-Au probe to identify subtypes of H1N1 and H3N2 viruses on an antibody array. Monoclonal antibodies (0.1 mg/ml) against pH1N1 and H3N2 viruses were spotted onto an Fc binding peptide-conjugated glass surface in triplicate, followed by treatment with either H1N1 or H3N2 virus (50 μg/ml). Then, the glass surface was treated with the protein G-GBP-Au probe-bound NP3 antibody specific for influenza virus nucleoprotein. Hybridized viruses were then detected with the naked eye after the signal enhancement of the probe (Fig. 2). We were able to detect a distinct signal on a microarray using the protein G-GBP-Au probe and thus demonstrated a fast and convenient method for identifying H1N1 and H3N2 subtypes. The detection limit of H3N2 using the protein G-GBP-Au probe was as low as 0.08 μg/ml (data not shown).

We further compared the efficiency of the proposed scanometric immunoassay to the fluorescence-based method on a glass slide spotted with various types of capture antibodies, including anti-H1N1, anti-H3N2, anti-H5N2, and anti-H1N2, in a microarray format. Influenza A/california/04/2009 (pH1N1) virus was applied to the glass surface and subsequently treated with the NP3 antibody specific for influenza virus nucleoprotein. Then, the surface was treated with a Cy5-labeled secondary antibody for fluorescence-based detection (Fig. 3a). By contrast, influenza

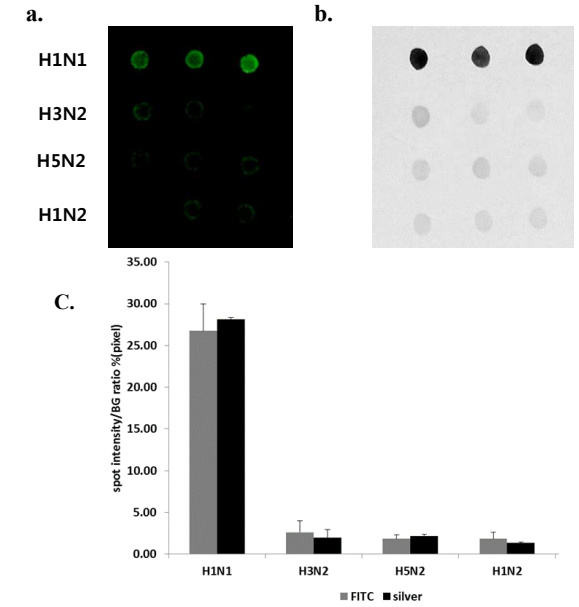


Fig. 3 Comparison of the relative average signal intensity for the fluorescence and scanometric detection methods. Each probe was spotted in triplicate.

A/california/04/2009 (pH1N1) on the slide was directly identified by the naked eye using the protein G-GBP-Au probe-bound NP3 antibody followed by signal enhancement (Fig. 3b). We were able to detect highly specific signals for influenza A/california/04/2009 (pH1N1) virus in both systems. In fact, the resulting signal intensities of the spots obtained in the images clearly demonstrate that the scanometric assay is sufficiently sensitive to provide a specificity for the target influenza virus similar to that of the fluorescence assay method (Fig. 3c).

In this study, we developed a tool for a novel, facile, and naked-eye visible immunoassay using a protein G-GBP-Au probe. The probe contains protein G to achieve high binding affinity with various types of antibodies (Fig. S3) and GBP to attain signal enhancement of the probe for detection with the naked eye or with an optical scanner. We demonstrated successful labeling of an antibody, and thus a target protein, with the probe. Importantly, the sensitivity of the scanometric immunoassay was comparable to that of the fluorescence-based method, allowing clear identification of the target virus. This scanometric immunoassay probe enables rapid analysis by directly labeling the primary antibody without a further incubation step with a secondary antibody. No chemical step is involved in labeling the antibody with the protein G-GBP-Au probe, thus greatly improving the efficiency of the immunoassay by creating a suitable antibody-probe complex for further antibody-antigen reactions, in contrast to conventional immunoassays. We expect that this probe will be useful in many applications, especially for point-of-care diagnostics for which ease of use, sensitivity, an ability to operate without special equipment, and low reagent costs are important factors.

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