Au nanoparticle- and silver-enhancement reaction-amplified microgravimetric biosensor

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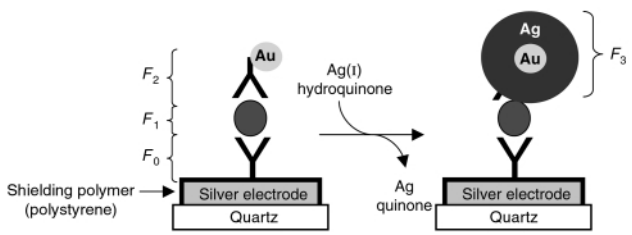
An ultrasensitive microgravimetric biosensor has been developed incorporating Au nanoparticle-amplified sandwiched immunoassay and silver enhancement reaction; Au nanoparticle-promoted silver(I) reduction and silver metal deposition result in *ca.* two orders of magnitude improvement in human IgG quantification.

The use of gold nanoparticles as a signal amplifier has attracted increasing interest in biosensor development. For example, in surface plasmon resonance[1–3] and microgravimetric quartz crystal microbalance (QCM)[4] based protein binding assay and DNA hybridization assay, mass coupling of functional Au nanoparticles results in a tens- to hundreds-fold sensitivity increase. However, the use of primary Au nanoparticles is not the end of the amplification. Efforts have been made to realize ultrasensitive biosensing by means of sequent amplification routes of, for example, multilayered[5] or dendritic-type Au nanoparticles.[6] In the latter example (microgravimetric DNA sensor), the primary Au nanoparticle amplification (*ca.* 10-fold) is followed by a secondary dendritic-type Au nanoparticle interaction. A total of 30-fold signal enhancement is achieved through the stepwise amplification.

Immunogold silver staining (IGSS) has been widely used in histochemical microscopy studies,[7] where functional Au nanoparticles act as catalysts to reduce silver ions to metallic silver. The autometallographic silver deposition procedure enlarges the size and darkens the color of the particles, such that protein-, antibody- or DNA-conjugated Au particles become visible under electron- or light-microscope. Most recently, this amplification strategy has been adopted in a pioneering study of scanometric DNA array quantification.[8] The incorporation of Au nanoparticle labeling and silver staining amplification results in two orders of magnitude improvement in detection sensitivity when compared with a conventional fluorophore system. Based on the similar amplification routes, a silicon-based flexural plate wave sensor has been developed.[9] The mass loading caused by Au particle-catalyzed deposition of metallic silver leads to a five orders of magnitude improvement in cancer antigen detection. In addition to the Au conjugate-promoted silver staining reaction, enzyme conjugate-catalyzed precipitation processes have also been applied for signal amplification in QCM and electrochemical sensors.[10,11]

Using an Au conjugate as a biocatalytic probe, we present herein an ultrasensitive microgravimetric QCM biosensor, in which primary Au nanoparticle-amplified sandwiched-immunoassay is followed by a silver staining reaction. The sensor elements are 10 MHz AT-cut quartz crystals coated with silver electrodes. An immunochemical molecular recognition event between anti-human IgG (a-h IgG) and human IgG (h IgG) is chosen to illustrate the sandwich procedure and, sequentially, the silver enhancement reaction (Scheme 1). It can be seen the proposed assay starts from the shielding of the metal electrode by polystyrene treatment. As reported in our previous study,[12] deposition of a polystyrene film (from a 5 mg ml^{-1} toluene solution) shields the silver electrode from undesirable oxidation and, in addition, provides a substrate for biomolecular immobilization. In this study, to evaluate the coverage of the polymer film further, the silver enhancement reagent [a freshly prepared 1:1 mixture of the silver enhancement solution A



Scheme 1 Schematic illustration of the Au nanoparticle-based, sandwiched immunoassay and silver staining amplification.

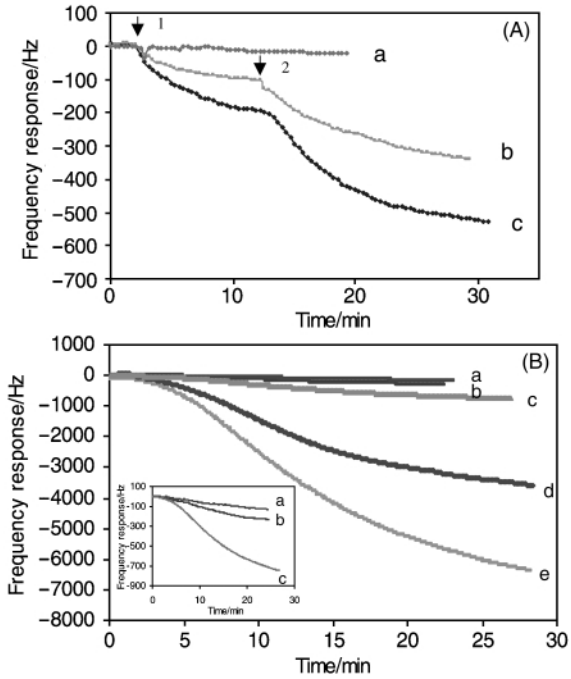


Fig. 1 (A) Frequency response of a-h IgG-modified sensors upon the application of h-IgG (10 μg ml^{-1}, curve b; 20 μg ml^{-1}, curve c) at arrow 1 and, sequentially, F_c specific a-h IgG:Au at arrow 2. Curve a is obtained when the modified sensor was exposed to the Au conjugate directly without h IgG application. (B) Frequency response of sandwiched complex coated sensors upon the exposure to silver enhancement reagent. Curves a and b are the background signals obtained in the absence of Au conjugate and h IgG incubation, respectively, in the sandwiched procedures; curves c, d and e represent h IgG concentrations of 0.5, 10 and 20 μg ml^{-1}, respectively.

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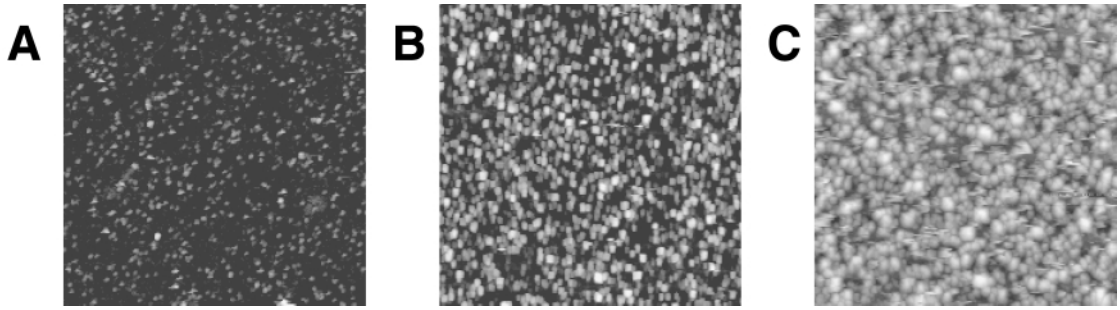

Fig. 2 AFM images (1 μm × 1 μm) of anti-h IgG/h-IgG (20 μg ml^{-1})/a-h IgG:Au coated surfaces before exposure (A), and after 10 min (B) and 30 min (C) exposure to silver enhancement solution.

(silver salt) and solution B (hydroquinone initiator) from Silver Enhancer Kit, SE-100 (Sigma)] was applied to the treated surface. After 30 min of reaction, no darkening of the surface was observed and the resonant frequency of the sensor remained unchanged. Thus it can be assumed that, after shielding by polystyrene, none of the metal electrode is exposed to serve as a nucleation site for silver ion reduction. This is essential for the proposed assay.

After polystyrene coating, the sensor surfaces were exposed to 0.5 mg ml^{-1} of a-h IgG (γ-chain specific, Sigma) in PBS buffer, pH 7.4. Through 1 h of incubation at 37 °C, the antibody molecules were firmly immobilized through passive adsorption and hydrophobic interaction. After blocking the non-occupied binding sites (2% BSA incubation for 15 min), the modified surfaces (with resonant frequency F_0) were exposed to h IgG (purified from serum, Sigma) and, when equilibrated, to 10 nm Au-conjugated a-h IgG (F_c-specific, Sigma). As a result, the analyte (h IgG) was sandwiched between bound antibodies and Au-conjugated antibodies. Fig. 1(A) shows the frequency responses of the sandwich procedures. The binding of h IgG with bound a-h IgG leads to a smooth frequency decrease to F_1. Complete binding is achieved within *ca*. 10 min. The following Au conjugate application results in further frequency response to F_2 after a longer duration of about 20 min. The total frequency changes caused by the sandwich procedures (F_0–F_2) are three- to four-fold greater than those obtained by direct analyte binding (F_0–F_1). The lower response of the Au conjugates may arise from steric inhibition of the huge molecules (it has been estimated that a 10 nm Au particle conjugates with seven IgG molecules). If the a-h IgG-modified sensor was exposed to Au-conjugated a-h IgG directly without analyte application, no frequency response was detectable [curve a in Fig. 1(A)]. This confirms that the binding of the Au-conjugate is attributable to the primary amplification for h IgG determination in the sandwich assay.

This is not the end of the amplification. Upon formation of the sandwiched immunocomplex, the sensor surfaces are coated with Au nanoparticles, which serve as nucleation sites to catalyze silver ion reduction. Fig. 1(B) shows the frequency responses of the sandwiched-immunocomplex-coated surfaces upon application of the silver enhancement solution. The deposition of metallic silver on the surface of the bound Au particle boosts the signal further to F_3 (Scheme 1). To optimize the signal and to reduce the silver staining background, after the sandwich reactions the sensors were rinsed thoroughly using deionized water to remove all salt content so as to avoid the auto-nucleation of silver. In addition, a five-fold diluted silver enhancement solution was optimal in reducing the staining background obtained in the absence of Au-conjugated antibodies [curve (a) in Fig. 1(B)] or h IgG [curve (b) in Fig. 1(B)]. These results confirm that neither significant nonspecific silver staining nor nonspecific Au particle binding occurs. Under the optimal conditions, the enormous frequency changes caused by silver metal deposition on a-h IgG/h IgG/a-h IgG:Au-coated sensors (F_2–F_3) are reflective of h IgG at different concentrations [curves c–e in Fig. 1(B)]. The incorporated primary and secondary amplified signals (F_0–F_3) are proportional to h IgG concentrations with ΔF(Hz) = $317C_{IgG}$(μg ml^{-1}) + 256, r^2 = 0.962. This is about two orders of magnitude more sensitive than that of direct assay.[12] It is also interesting to note that Fig. 2(B) reveals the mechanism of Au-promoted silver(I) reduction: within the first 5 min, the reduction is slow; after 5 min, significant reduction appears; and at *ca*. 30 min the reduction tends to be complete.[7]

The above observations are further proved by atomic force microscopy (AFM) studies. AFM images(1 μm × 1 μm) acquired for a-h IgG/h IgG/a-h IgG:Au-coated surfaces before [Fig. 2(A)] and after exposure to the silver enhancement solution [Fig. 2(B) and 2(C)] were obtained using a TMX 2000 Explorer System (TopoMetrix, Santa Clara, CA) operated under ambient conditions. Si_3N_4 cantilevers with V-shaped tips (20–50 nm radius of curvature) were used for measurement in contact mode. Although the observed particle size may deviate from the actual value because of the intrinsic curvature of the tip,[13] the deposition of metallic silver on the surface of the Au particles is still clearly evident.

In conclusion, we have developed an ultrasensitive QCM biosensor which combines metal electrode shielding, Au nanoparticle-amplified sandwiched-assay and a silver enhancement reaction. Based on this method, the detection sensitivity of the microgravimetric sensor is no longer subject to the mass of the analyte molecules. Since the proposed assay relies on the shielding of the metal electrode, the advanced electrode shielding technique remains a challenge. For example, functionalization of a polystyrene film may combine electrode protection with covalent DNA molecule immobilization in one preparation step, such that the proposed assay can be extended to ultrasensitive DNA analysis. In addition, this study suggests the possibility of utilizing the silver enhancement reaction for quantification in chip-based biosensing.

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