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RESEARCH PAPER

Surface chemical approach to single-step measurement of antibody in human serum using localized surface plasmon resonance biosensor on microtiter plate system

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Abstract In clinical settings, serum antibody levels serve as markers of pathology. For example, antibodies related to autoimmune diseases are among the conventional targets in laboratory tests. Simple clinical tests can improve the efficacy of laboratory practice. This study describes a single-step, wash-free technique for optically detecting antibodies in human serum through the localized surface plasmon resonance (LSPR) of gold nanoparticles. As a proof-of-concept experiment, the amount of antibiotin dissolved in human serum was measured with a LSPR-based biosensor in a wash-free manner using a conventional 96-well microtiter plate and a plate reader. For an efficient surface modification of biosensors, zwitterionic copolymer was used as a scaffold on the gold nanoparticle surface to immobilize antigen and blocking reagent. Single-step, wash-free measurement of antibiotin in human serum was successfully achieved. In addition, nonspecific responses from serum contents were significantly reduced because both the copolymer and hydrophilic antigen reagent that we employed were composed of poly(ethylene oxide) spacer. Comparative experiments of the antigen-antibody reaction in serum to that in buffered solution revealed that serum is a favorable environment for the biological reaction. In conclusion, our gold-nanoparticle-based LSPR method may provide a rapid and simple way to measure the amount of antibody in serum quantitatively in clinical practice.

Keywords Single-step immunoassay · Human serum · Biosensor · Localized surface plasmon resonance (LSPR) · Microtiter plate system · Laboratory test

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Introduction

In the emerging nanotechnology field, metal nanoparticles have been the focus of much research due to their unique optical properties [1]. In particular, noble metal nanoparticles have generated considerable interest for their biomedical applicability in clinical practice because of their stability and compatibility with the size of biological building blocks, such as proteins and DNA [2]. An increasing number of studies in biological sensing [3–15], clinical diagnostics [16–19], and medical therapy [20–22] have been reported.

The unique optical properties of metal nanoparticles originate from their conduction electrons, also known as surface plasmons, at the metal/dielectric interface. When excited by light, surface plasmons exhibit collective oscillations of electrons that result in the absorption and scattering of incident light. This phenomenon, called localized surface plasmon resonance (LSPR), distinguishes the metal nanoparticles from the bulk material [23, 24]. Gold and silver nanoparticles elicit the intense extinction of incident light, which can be physically tuned by changing the particle shape, size, and interparticle distance [11, 25–28].

The characteristic LSPR absorption spectrum is extremely susceptible to dielectric properties of the local environment around metal nanoparticles [26, 29]. This property has attracted interest for the development of biosensors based on LSPR. The critical volume which influences the shape and position of the

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LSPR spectra ranges from several tens to several hundreds of nanometers from the nanoparticle surface. Target molecules, such as antibodies, antigens, DNA, and so on, can be captured within this critical volume. By measuring the extinction spectral changes, we can estimate the corresponding amount of target molecule bound to the nanoparticle surface.

In the past decade, many theoretical and fundamental works have employed LSPR as a biosensing transducer. Malinsky and coworkers [8] explored the sensing capabilities of LSPR by changing the thickness of alkanethiol self-assembled monolayers formed on silver nanoparticles. Using colloidal gold immobilized on an amino-functionalized glass substrate, Nath and Chilkoti [10] demonstrated a simple, label-free optical biosensor application of LSPR. Haes and coworkers [17, 18] used lithographically fabricated nanoparticles to quantify a biomarker for Alzheimer's disease. Since our focus is the application of LSPR in clinical practice, we previously studied the optimal arrangement of immobilized colloidal gold to detect antibodies in test solution with a standard plate reader and 96-well microtiter plates [30]. These experiments were conducted in a buffered solution to remove any possible artifacts. Recently, Wang et al. [19] reported LSPR sensing of the streptavidin-biotin reaction in diluted human blood.

Our group also reported a single-step, wash-free method to measure antibody in human serum using immobilized colloidal gold on microtiter plates [31]. It enabled rapid quantification of blood group ABO-related immunoglobulin levels after living-donor organ transplantations, which are critically essential for treatments and prognosis of transplantees especially after clinical ABO-unmatched transplant. Because conventional immunoassays are laborious and time-consuming, we are interested in developing a rapid, simple, and reproducible analytical method for clinical practice. We expect this LSPR-based single-step, wash-free method can serve as such a simplified assay for a specific analyte in human serum. Here, we further explore the analytical validity of our LSPR sensor for the antigen-antibody reaction in human serum comparing to that in a buffered solution. Our previous results were obtained by measuring naturally produced antibody in human serum. On the other hand, in the present study, we employed the model reaction of antibiotin to enable us to chemically distinguish the reactions of the target substance from the unknown serum contents. Using the controlled extrinsic antibody was necessary here because the primary purpose of the present study was validated reduction in nonspecific reactions of serum. In the crude systems like serum we used here, nonspecific reactions are major factor to deteriorate sensor sensitivity.

First, we optimized the surface chemistry to improve signal intensity. The LSPR sensor is a label-free method. To increase signal intensity, nonspecific responses from unknown species in the serum must be reduced or eliminated. Second, to facilitate the antigen-antibody reaction, we compared the reactions when hydrophilic or hydrophobic spacers were attached to the immobilized antigen molecules (biotin). We also compared the reactions in buffered solution and human serum because we believe that some biological interactions should occur in their natural biological fluid. Owing to the improved signal, the final measurements would be obtained in a wash-free manner without substituting a buffered solution for the serum.

Materials and methods

Chemicals and human samples

The following materials were obtained from their respective manufacturers: colloidal gold (100 nm diameter) (BBInternational); polyampholite polymer PAS-410 (Nitto Boseki); rabbit antibiotin (Rockland Immunochemicals); *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Sigma-Aldrich); (+)-biotin-$(PEO)_4$-amine (PEO_4-biotin), (+)-biotinyl hexylamine (hexyl-biotin), and triethylene glycol monoamine (TEA) (Molecular BioSciences); *N*-hydroxysuccinimidobiotin (NHS-biotin), succinimidyl-6-(biotinamido)-6-hexylamidohexanoate (NHS-LC-LC-biotin), NHS-PEO_4-biotin, and NHS-PEO_{12}-biotin (Pierce Biotechnology); fatty acid-free bovine serum albumin (BSA) (Merck); skim milk powder and 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 4.7) buffered saline (Wako Pure Chemical Industries); and phosphate-buffered saline (PBS, pH 7.6) (Lab Vision). In all experiments, reagent-grade water obtained from a Milli-Q system (Millipore) was used.

Serum samples were obtained from a healthy adult volunteer at Kyoto University Hospital with informed consent according to the Declaration of Helsinki. Once the serum was separated from blood cells, it was stored in a −20 °C freezer until use.

Immobilization of gold nanoparticles onto microtiter plates

Amine-surface 96-well microtiter plates (Corning) were used as the substrates on which the gold nanoparticles were immobilized (Fig. 1c). The optimal concentration of the gold nanoparticle solution (about 1.7×10^9 particles/μL) was incubated in the wells at room temperature for 24 h to allow the particles to immobilize on the well surface [30]. After incubation, the plates were shaken vigorously to remove all moisture and washed by ultrapure water, then thoroughly dried with nitrogen gas. For each well, the quality of immobilization was checked by measuring the peak position of the extinction spectrum.

Functionalization of the immobilized gold nanoparticle surface

Two different surface chemistries were employed to immobilize antigen on the gold nanoparticle surface (Fig. 1). The first, simpler method used biotinylated BSA (Fig. 1a). The BSA was biotinylated by using a series of succinimidylated biotin reagents, following the manufacturer's instructions. In all cases, a 12-fold molar excess of biotin (relative to BSA) was prepared. Four different types of spacers (i.e., biotin, LC-LC-biotin, PEO_4-biotin, and PEO_{12}-biotin) were used as the biotin reagents. Those spacers were chosen according to the results of Frederix et al. [32], who showed that hydrophilic PEO spacers reduce nonspecific adsorption from IgG in the antigen-antibody reaction.

Biotinylated BSA was attached to the immobilized gold nanoparticles by incubating 10 mg/mL of biotinylated BSA in PBS for 2 h at 30 °C. The BSA covered the surface of the gold and plate, and also served as an antigen for antibiotin. Reference wells were prepared by applying the same procedure with nonbiotinylated BSA. To block the nonspecific binding of unknown serum contents onto the gold nanoparticles and plate surface, 0.025 % skim milk solution was added and incubated for 2 h. The functionalized plates were stored with 100 μL of the blocking solution at 4 °C for further use.

The second method used PAS-410, which is a water-soluble zwitterionic copolymer comprised of diallylamine hydrochloride salt and maleic acid (Fig. 1b). This polymer worked as a scaffold layer to attach antigen molecules chemically to the immobilized gold nanoparticles through its carboxyl groups. The polymer amine groups served as functional components to coat the nanoparticles via their strong electrostatic interaction with gold. The optimal PAS-410 concentration (8.4×10^{-4} % (*w*/*w*)) at pH 2.0 was added to each well and incubated for 30 min.

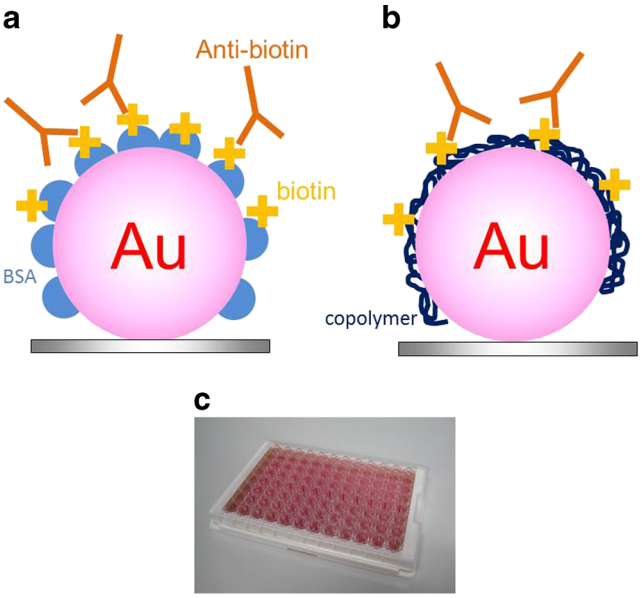

Fig. 1 Experimental setups illustrating two different methods of surface chemistry to functionalize the gold nanoparticle surface. The nanoparticle surface was coated with (**a**) biotinylated BSA or (**b**) a zwitterionic copolymer. (**c**) A 96-well microtiter plate with immobilized gold nanoparticles. The amount of antibiotin that attached to biotin on the surface was measured by the peak shift of LSPR spectra

Aminated antigen molecules (i.e., PEO_4-biotin and hexyl-biotin) were immobilized onto the surface of the gold nanoparticles. Immobilization was accomplished through the amide linkage formed between the amine groups of the antigen molecules and the carboxyl groups of PAS-410 by using the EDC coupling reagent. Reference wells were prepared by applying the same procedure with complementary molecules of TEA, which are structurally similar to the antigen molecules. The dehydration reaction was initiated by adding 25 mL aliquots of 10 mM antigen solution and 200 mg/mL EDC solution in MES simultaneously to each well while maintaining the temperature at 37 °C for 1 h. Lastly, to block nonspecific binding of unknown serum contents onto the gold nanoparticles and plate surface, 0.01 % BSA solution was added and incubated for 2 h. Functionalized plates were stored with 100 μL of the blocking solution at 4 °C for further use.

LSPR measurement of the amount of target antibody (antibiotin) on the surface of immobilized gold nanoparticles

The LSPR sensor was characterized by determining shifts in the spectrum peak (λmax), which reflect changes in the refractive index at or near the gold nanoparticle surface. The change in the refractive index results from the specific binding of antibiotin to biotin.

Antibiotin was diluted in PBS containing 0.1 % BSA (for the biotinylated BSA method) or 0.26 % PAS-410 and 0.01 % BSA (for the copolymer method). This mixture was combined with crude human serum to prepare the test solution. The serum content was one third of the test solution. A 100 μL aliquot of test solution was incubated in each well for 30 min at room temperature. At first, the optical extinction spectrum in the "wash-free" condition was measured with the test solution remaining in the well. Then, each well was washed four times by replacing the test solution with PBS, and the optical extinction spectrum was measured again in 100 μL of PBS in the "wash" condition. The specific shift in λmax, which is induced by the binding of antibiotin to immobilized antigens, was computed by subtracting the peak shift of the simultaneously measured reference well. A plot of the concentration-dependent shift in λmax was created to compare the responses under the two measurement conditions.

Apparatus

A plate reader (Varioskan, Thermo Fisher Scientific) was used to measure the optical extinction spectra of immobilized gold

nanoparticles on the plates. The plate was kept at room temperature throughout the measurement procedure. Extinction spectra were collected from 540 to 610 nm with 1-nm steps. Spectral data were analyzed by fitting them to a Gaussian curve to identify λmax. This analysis was conducted with IGOR Pro software (WaveMetrics).

Results and discussion

Biotinylated BSA method for presenting antigens

Biotinylation of BSA was completed with the help of efficient succinimydylated biotin reagents. Since excess succinimidylated biotin reagents were used, it was assumed that the BSA biotinylation was saturated and constant across all spacer types. Even if excess nonreacted and hydrolyzed reagents possibly remained in the BSA solution, they were easily washed out after biotinylated BSA was attached to the gold nanoparticles.

The binding of antibiotin to biotin was measured with a plate reader. Representative spectra are given in Fig. 2. As shown in Fig. 3a, consistent results were obtained for the four different spacer types for the reaction in PBS. In contrast, a slightly higher response was observed in the case of more hydrophilic poly(ethylene oxide) (PEO) spacers for the reaction in serum (Fig. 3b). Because we could not completely eliminate the effect of nonspecific binding in the lower concentration range of antibiotin, even after subtracting the response in the reference well (Fig. 3b), we concluded that the biotinylated BSA method could not be used satisfactorily with serum samples.

The sigmoidally fitted curves in Fig. 2 indicate that the dissociation constants (Kd) of this assay with PEO_{12} were 1.0×10^{-6} and 2.7×10^{-7} M in PBS and serum, respectively. Thus, the reaction seems more efficient in serum than in PBS.

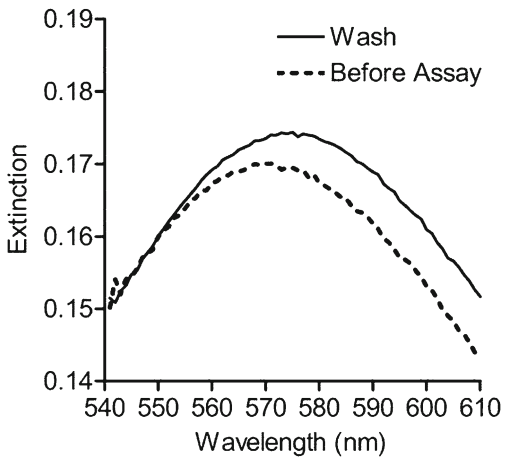

Fig. 2 Representative spectra of LSPR in PBS, obtained by using the copolymer method with a PEO_4-type spacer. *Solid line* "wash" measurement, *dotted line* baseline before assay

Copolymer method for presenting antigens

To improve the sensing performance with serum samples, we evaluated the second method employing a zwitterionic copolymer rather than biotinylated BSA as a scaffold on the gold nanoparticles. Copolymer was adsorbed onto the gold nanoparticles through the electrostatic interaction between its amino groups and gold. Then, two kinds of biotin antigens, PEO_4-biotin and hexyl-biotin, were covalently immobilized on the surface of the gold nanoparticles, with the help of the EDC coupling reagent. The binding of antibiotin to biotin was monitored through measuring the extinction spectra.

According to the results shown in Fig. 4, PEO_4-biotin performed much better than hexyl-biotin in both PBS and serum. This observation was consistent with that of biotinylated BSA. Hydrophilic spacers are more favorable for the reaction than hydrophobic ones, even assuming that the extended spacer length of both compounds is sufficient to reduce steric interferences. In the copolymer method, the serum measurements in the lower concentration range were almost zero, as expected (Fig. 4b). Therefore, its blocking capability seems to be higher than that of the biotinylated BSA method. In addition, we observed greater reaction responses in serum than in PBS. For instance, in the case of PEO_4-biotin, the maximum peak shifts observed at 5 mg/mL of antibiotin were 3.9 and 5.3 nm in PBS and serum, respectively. However, the standard error was only slightly increased, from 0.11 to 0.13 nm. As hypothesized, for the antigen-antibody reaction, a biological fluid such as serum appears to be a more appropriate environment for measurements than a buffered solution.

The observed absolute values of peak shift were smaller than those for biotinylated BSA method (Fig. 3), probably due to the difference in the density of immobilized biotin antigens between the two methods. However, the Kd obtained in serum with PEO_4-biotin was 6.7×10^{-8} M, which is smaller than the value observed with the biotinylated BSA method. This result reflects the improved sensitivity of the copolymer method as a biosensor. The reduction in the nonspecific responses from serum samples improved the sensitivity in the case of PEO_4-biotin. Using a silver nanoparticle technique, Hall et al. [33] reported a Kd value of approximately 3×10^{-7} M, which is similar to ours.

Single-step, wash-free measurement of serum samples

We applied our assay to a single-step measurement using the copolymer method to functionalize the gold nanoparticles. The single-step measurement is "wash-free" because we do not discard the serum in each well before measuring the spectrum. The major purpose of "washing" is to reduce the signal from unknown species in the serum and to improve measurement accuracy and sensitivity. Therefore, a very low nonspecific response or large SNR must be achieved for the "wash-free" measurement to be useful.

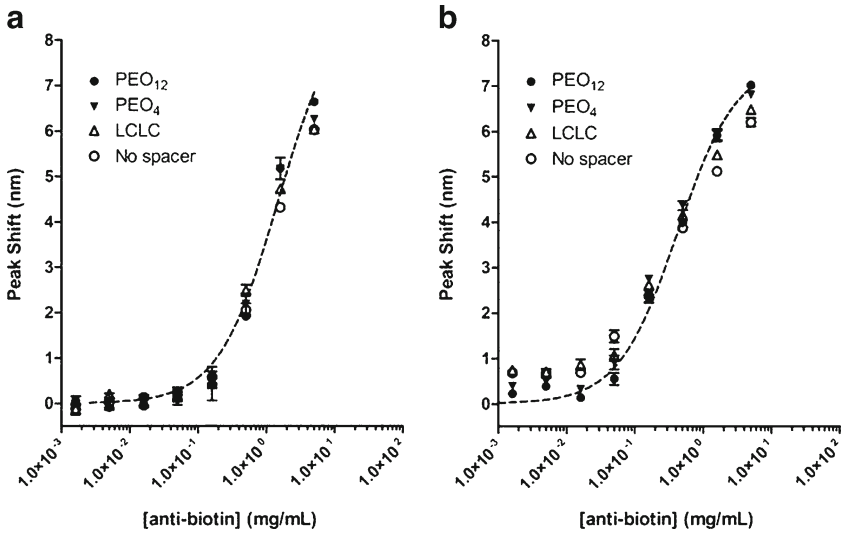

Fig. 3 Antibiotin concentration-dependent shift in λmax obtained with different spacers in antigen molecules (i.e., *filled circle* PEO_{12}, *filled triangle* PEO_4, and *open triangle* LCLC) or no spacer (*open circle*). The biotinylated BSA method was used to immobilize antigen on the gold nanoparticle surface. Measurements were done (**a**) in PBS and (**b**) in serum. *Dotted line* delineates the responses of PEO_{12}. *Error bars* represent standard errors from four samples

The "wash-free" measurements in PBS and serum (Fig. 5) resembled the results in the "wash" condition (Fig. 4). The antigen-antibody reaction was equally enhanced with PEO_4-biotin and serum compared to hexyl-biotin and PBS, respectively. One-to-one comparisons between the "wash" (Fig. 4) and "wash-free" (Fig. 5) results showed similar concentration-dependent responses of the LSPR peak shifts for each spacer type of biotin. A slightly larger variability was found in the "wash-free" measurement, due to the unpredictable and uncontrolled adsorption of serum contents (standard errors, 0.08 and 0.17 nm for PBS and serum with PEO_4-biotin, respectively at 0.16 mg/mL of antibiotin). However, our results indicate that it is quite feasible to apply this single-step, wash-free measurement to both PBS and serum samples.

Combined with our previous results, the findings presented here support the validity of our single-step, wash-free LSPR assay for antibody quantification in human serum using a standard plate reader and 96-well microtiter plates. In this method, the nonspecific binding was significantly reduced and similar responses were obtained in the "wash" and "wash-free" assays, even in human serum. Thus, the method may provide a rapid and simple way to measure the amount of antibody in serum quantitatively in clinical practice.

There is an analytically important issue that arises with the use of serum. The absorption of serum distorted the shape of the LSPR spectrum, with a negligible change in λmax. This distortion leads to a deviation from the Gaussian curve, due to the slightly yellowish color of serum. Therefore, one must be

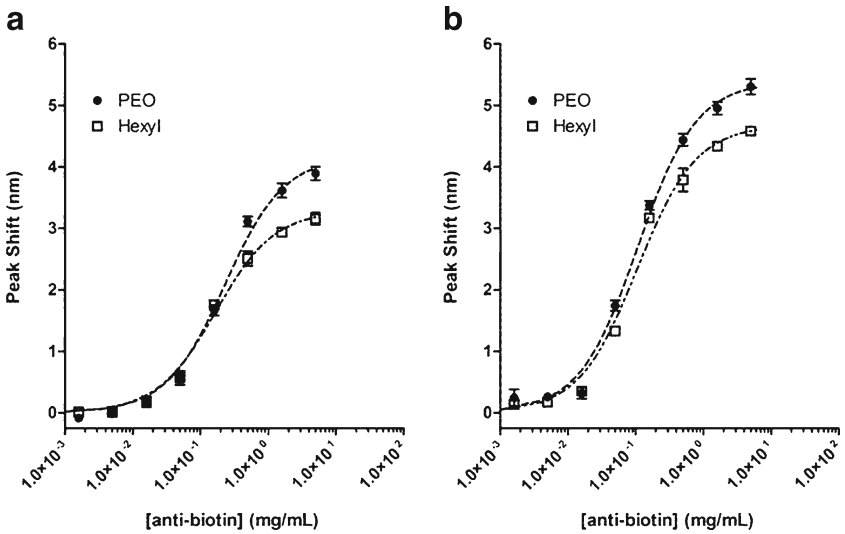

Fig. 4 Antibiotin concentration-dependent shift in λmax obtained with different spacers (i.e., *filled circle* PEO_4-type spacer and *open square* hexyl-type spacer) in antigen molecules. The copolymer method was used to immobilize antigen on the gold nanoparticle surface. "Wash" measurements were done (**a**) in PBS and (**b**) in serum. *Error bars* represent standard errors. Limits of detection are 0.016 and 0.005 mg/mL for PBS and serum, respectively. Limits of quantification are 0.016 and 0.05 mg/mL for PBS and serum, respectively, derived from our coarse measurement scales

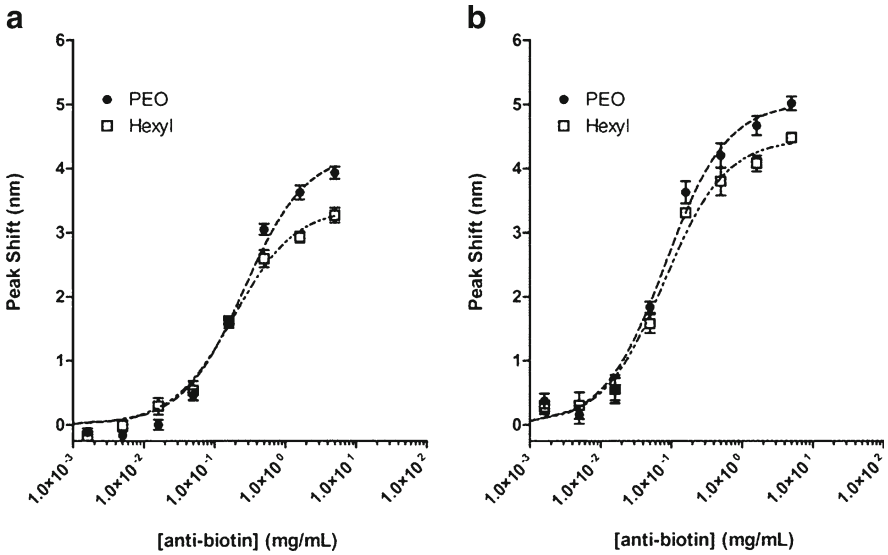

Fig. 5 Antibiotin concentration-dependent shift in λmax obtained with different spacers (i.e., *filled circle* PEO_4-type spacer and *open square* hexyl-type spacer) in antigen molecules. The copolymer method was used to immobilize antigen on the gold nanoparticle surface. "Wash-free" measurements were done (**a**) in PBS and (**b**) in serum. *Error bars* represent standard errors. Limits of detection are 0.016 and 0.005 mg/mL for PBS and serum, respectively. Limits of quantification are 0.016 mg/mL for both PBS and serum, respectively, derived from our coarse measurement scales

cautious and take the spectroscopic effects of the serum into account when "wash-free" measurements are used.

Limitations of our study

The sensitivity of our method is not confirmed high enough to measurer antibody related to infectious diseases such as anti-HBs or antitetanus toxoid antibody. Therefore, further studies are necessary to expand applicability of our methods. For instance, hepatitis B test is to be proposed using the combination of recombinant hepatitis B surface antigen and anti-HBs. Moreover, another limitation of our methods would be that test solution was diluted serum with PBS containing controlled antibody and blocking reagent. It should also be investigated to accept test solution with higher serum contents.

Conclusions

Superior surface chemistry using a zwitterionic copolymer and hydrophilic spacer arms improved the simple operability of a label-free LSPR biosensor, which eventually led to a single-step procedure based on a microtiter plate system by significantly reducing nonspecific responses of crude serum. We showed the method which quantitatively measures the amount of antibody in human serum with this single-step procedure as in PBS. Moreover, the antigen-antibody reaction in human serum was more efficient than the reaction in a buffered solution. Consequent "wash" and "wash-free" measurements gave equivalent results in terms of the concentration-dependent shift in λmax, due to the reduction in nonspecific responses by our sensor preparation. These results were achieved by using a novel copolymer scaffold to immobilize both the antigen and the blocking agent. The spacer arm of the antigen was optimized by comparing hydrophilic PEO spacers with hydrophobic alkyl spacers. Use of a hydrophilic spacer was superior for the reaction with the antibody, i.e., antibiotin.

On the other hand, conventional techniques, such as enzyme-linked immunosorbent assay (ELISA), require a label (such as peroxidase) to transduce analytical information into an observable signal (such as a color change). These techniques involve several steps and hours to complete. Additionally, procedures such as replacing test solution and washing wells are intrinsically subject to human error-based variability in the measurements.

This paper reports proof-of-concept results of single-step, wash-free measurements of antibody in human serum based on the LSPR biosensor using a microtiter plate system. Combination of surface chemistry and spacer arm selection gave us the feasible condition in preparation of the LSPR biosensor by reducing nonspecific responses. We successfully measured the amount of antibiotin in serum as in a buffered solution. We confirmed analytical validity of our method. The results also show the importance of measuring biomolecular interactions in an appropriate biological fluid, i.e., serum for the antigen-antibody reaction. Therefore, "wash-free" could be a critical characteristic for such analytical method to reflect the real biomolecular interactions. In principle, our analytical method is broadly applicable to various antigens other than biotin. Lastly, potential usefulness of the LSPR method in clinical practice as a laboratory test was clearly suggested here owing to its rapidness and simplicity of the procedure.

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