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Single-step, label-free quantification of antibody in human serum for clinical applications based on localized surface plasmon resonance

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Abstract

The amount of antibody in blood is an important measure of health status for making critical decisions in clinical practice. Here, we demonstrated a single-step, label-free, molecular diagnositic method based on localized surface plasmon resonance (LSPR) using standard 96-well microtiter plates. We improved the LSPR biosensor so that it can measure antibodies to blood group antigens in human serum with a single-step operation. First, we employed the ampholytic polymeric surface modification technique to present an efficient molecular scaffold on the sensor surface. Second, we selected the combination of an appropriate reference molecule against the antigen and a blocking agent to significantly reduce the variability of signal due to nonspecific responses of the unknown in the sample. Finally, we overcame the analytical difficulty arising from serum and achieved a single-step "wash-free" measurement of the amount of target antibody in human serum.

From the Clinical Editor: In this paper, a novel, single-step, label-free, molecular diagnositic method is discussed for antibody detection based on localized surface plasmon resonance using standard 96-well microtiter plates.

Key words: Optical biosensor; Localized surface plasmon resonance; Immunoassay; Blood test

In clinical tests, antibody titers in blood are very important measures for diagnosis. Antibodies related to infectious diseases such as chickenpox or hepatitis are among the conventional targets of those tests. In autoimmune diseases such as rheumatoid arthritis or systemic lupus erythematosus, autoantibodies play an important role in diagnosis as well. Other examples like blood group ABO-related immunoglobulin levels after living-donor organ transplantations are critically essential for treatments and prognosis of transplantees because antibody-mediated rejection is one of the primary causes of poor outcomes.[1] Several methods are used to measure antibody levels in serum. They include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence-activated flow cytometry (FACS). Moreover, among the various new types of chemical and biological sensors, the optical method using surface plasmon resonance (SPR) on planar gold films has made a particularly important contribution to affinity-based biosensing applications, including antigen-antibody reactions, during the past two decades. As mentioned in the reviews,[2-4] the SPR sensor has been widely used to monitor the change in the refractive index induced by a broad range of analyte bindings in a label-free manner. This direct detection method of biomolecules eliminates conventional signal transducers such as fluorophore and enzyme.

In our previous work,[5,6] we showed that the SPR method could be used to measure anti-human blood group A (anti-A) IgG titers in sera rapidly and quantitatively while avoiding the interference from IgM-type antibodies. This could be a promising tool because one of the clinical interests is the amount of IgG-type antibodies. Measurement of anti-A IgG titers in sera has been routinely conducted by the conventional test tube (TT) method. This method, however, has the intrinsic problem of interexaminer variability because it relies on visual observation.[7] Our study was the initial trial for clinical use of the SPR method.

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It improved the diagnostic operation by intermittently monitoring the level of the same patients' antibodies after clinical ABO-unmatched transplant.

However, because the SPR method is based on flow-cell type sensor chips, the resultant sequential operation is very complex and a more efficient procedure is desired in clinical practice. This instrumental characteristic possibly limits the reduction of the total assay time and the achievement of a high-throughput assay system. In addition, the SPR method detects unknown species, not only in the vicinity of the sensing planar gold surface, but at a relatively long distance from its surface. This can cause unpredictable and unnecessary responses of signals that affect the accuracy of the measurement and mandate the temperature control of the whole system to minimize the fluctuation of the refractive index. Therefore, signal compensations such as a reference flow cell or subsequent washing are necessary. Moreover, it requires us to regenerate a sensor chip surface before another test is to be done. It is quite a complex process to prepare the new sensor chip surface with the same sensor characteristics as previous ones. From the analytical viewpoint, these properties are not favorable to control the quality of each measurement in the long run.

In this article, inspired by the similarity to the SPR method and its less practical applicability, we further explore the possibility of the application of the localized surface plasmon resonance (LSPR) method[8] to this kind of measurement. As one of the promising technologies to acquire the information of molecular binding events, this method can be made with very simple set-up and assay procedures in comparison with those of SPR. Through the advance of nanofabrication techniques and nanophotonics, this particular label-free immunoassay has been widely investigated for many biological applications.[9-14] Previously, we have demonstrated its simple fabrication in a 96-well microtiter plate format and its applicability to a label-free immunoassay with the expectation that this method can be used in clinical tests.[15] We have found the density of immobilized gold nanoparticles (AuNPs) best to detect antibody in sample solution.

When metal nanoparticles are excited by light, their conduction electrons on the surface, known as surface plasmons, exhibit collective oscillations. These oscillations result in both absorption and scattering of incident light of a specific resonant wavelength. This phenomenon is called LSPR. The characteristic resonance energy of the surface plasmon is strongly dependent on the dielectric properties of the local environment around the nanometric particles.[16,17] Thus, small changes in the refractive index induced by surrounding liquid or analyte binding at or near the surface of nanometric particles can be measured as shifts in the LSPR spectra. We believe that the LSPR method has the benefits of both SPR and ELISA. These include a label-free simple assay like SPR and a high-throughput parallel operation like ELISA.

To our knowledge, our investigation is the first clinical application of the LSPR method with human blood samples. We measured anti-A IgG levels in human serum based on LSPR to compare with the previously established methods by SPR. Our study focused on simplicity, reproducibility and accuracy with respect to the clinical diagnosis. One of the difficulties in the label-free methodology is reducing the nonspecific responses from serum to increase the intensity of specific response. For this purpose, we employed novel zwitterionic copolymer to serve as a molecular scaffold on the surface of AuNPs. It can adsorb strongly onto the gold surface with static ionic interaction between its amino groups and gold and present efficient grafting points for further chemical modification on the surface of NPs, such as immobilization of antigen groups or blocking agents through its carboxyl groups, which would eventually lead to the improvement of signal intensity. Due to the improved signal intensity, the final measurements can be done even without removing and washing the serum before the measurements. We call this the "wash-free" measurement that simplifies the process as a single-step assay of a specific analyte in serum. Putting the test solution into a reaction well could be the only required operation to achieve accurate diagnostic results. Finally, for validation of our method, comparison of the results with several other diagnostic methods was also performed.

Methods

Functionalization of the surface of AuNPs

Ninety six-well polystyrene amine surface microtiter plates (Corning, Corning, New York) were used as the substrates on which the AuNPs (100 nmϕ; BBInternational, Cardiff, United Kingdom) were immobilized at the optimized density.[15] The surface of AuNPs were functionalized with polyampholite polymer PAS-410 (Nitto Boseki, Fukushima, Japan) and human blood group A trisaccharide antigen molecules (Carbohydrate Synthesis, Oxford, United Kingdom). Details in preventing nonspecific adsorption are described in the Supplementary Material, available online at http://www.nanomedjournal.com.

LSPR measurement of the levels of target antibody (anti-A)

Serum samples were taken from 41 healthy adult volunteers at Kyoto University Hospital, including 21 men and 20 women with informed consents according to the Declaration of Helsinki. Crude human serum was diluted by one third, using a diluent containing 0.26% PAS-410 and 0.01% BSA in phosphate buffered saline (PBS) (pH 7.0) to prepare the test solution. Without dilution, some samples were within the range of saturation of signal. Therefore, we decided to dilute all crude samples in this work. The diluent consisted of components similar to those on the surface of AuNPs, and we aimed to avoid unnecessary interaction between the sample and sensor surface. A 100 μL aliquot of the test solution was then incubated for 30 minutes at room temperature (24–25°C). Next, we measured its optical extinction spectrum in the "wash-free" condition. Subsequently, each well was washed and filled with 100 μL PBS. The optical extinction spectrum was measured again in the "wash" condition. The specific peak shift induced by the antibodies bound to immobilized antigens was obtained by subtracting the peak shift of the reference-well measured at the same point in time. With the purified anti-A IgG diluted in PBS, we created a plot of concentration-dependent shift of spectrum peak (λ_{max}) to estimate concentrations of the antibodies in the test solution. In addition, a

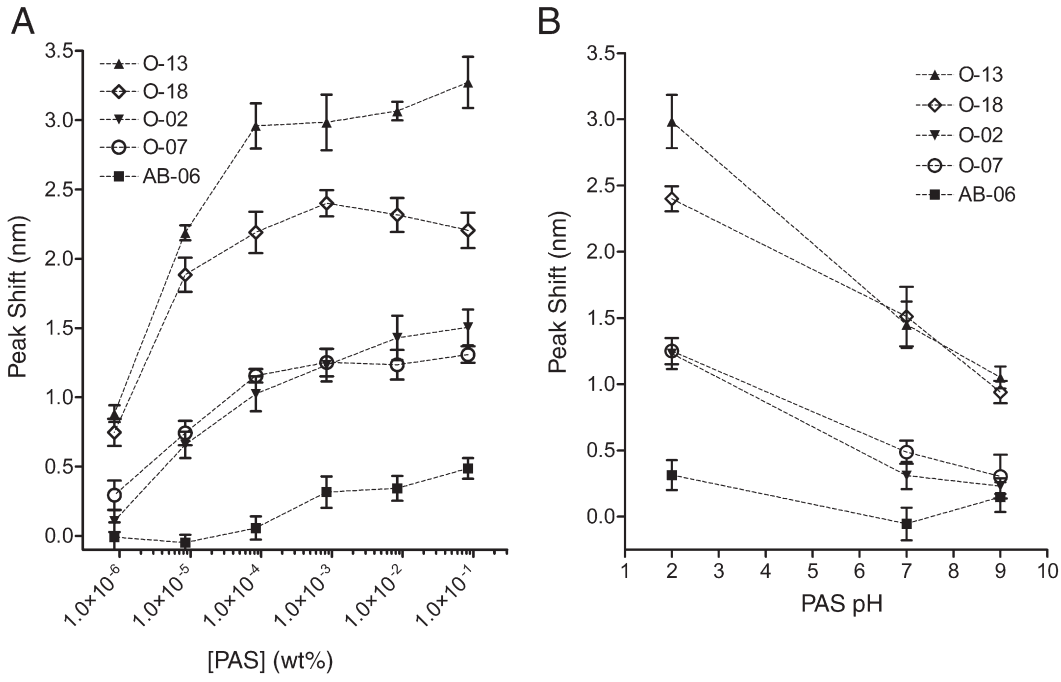

Figure 1. Effect of coating polymer concentrations and pH levels on sensor responses (spectrum peak (λ_{max}) shift). **(A)** Effect of concentration. **(B)** Effect of pH level. Data was acquired using the sera from 4 blood group O and 1 blood group AB volunteers. Error bars represent standard errors.

secondary analysis was performed to quantify bound IgG-type antibodies by adding a 100 μL aliquot of 100 μg/mL goat anti-human IgG monoclonal antibody (Rockland Immunochemicals, Gilbertsville, Pennsylvania) and incubating for 30 minutes at 37°C. The final spectra measurement was performed after being completely dried with compressed dried air. A plate reader (Varioskan; Thermo Fisher Scientific, Waltham, Massachusetts) was used to measure the optical extinction spectra of immobilized AuNPs on the plates. The spectra data were analyzed by fitting them to a Gaussian curve to identify a spectrum peak (IGOR Pro; WaveMetrics, Portland, Oregon).

Determination of antibody levels by the TT and SPR method and their correlation with those measured by the LSPR method

We employed the same procedure described elsewhere for the TT and SPR measurements.[5,6] For the SPR method, we used Biacore X system (GE Healthcare, Amersham, United Kingdom). The blood group A trisaccharide antigen molecules were immobilized on the sensor chip CM5 following the standard procedures. An anti-human IgG monoclonal antibody (Sanbio, Uden, The Netherlands) diluted with the running buffer HBS-EP (GE Healthcare) was used to measure the amounts of anti-A IgG associated with the blood group antigen A immobilized on the chip. All measurements were performed at 25°C. The deviations among different sensor chips were adjusted using the responses from the identical serum sample. We performed a statistical evaluation of correlation of the results from 3 methods. We computed linear correlations of both the TT and SPR methods with the LSPR method (SAS 9.1 software; SAS Institute, Cary, North Carolina).

Results

Sensor functionalization and assay optimization

Our sensor elements consisted of monodisperse AuNPs, which were immobilized on the surface of polystyrene microtiter plates as reported in our previous work.[15] Microtiter plates are very useful for multiplexing the assay on the same plate. Fundamentally, our LSPR method is characterized by determining shifts of λ_{max}, which are the measure of changes in the refractive index at or near the proximity of the surface of AuNPs. These shifts are induced by specific analyte binding to target elements on the surface of AuNPs and are to be converted into the actual amount of analyte molecules bound on the surface using the standard response curve. Spectrum measurement was done by standard plate reader. The surface of AuNPs was first coated with the polyampholytic polymer anchored on their surface by its amino groups. The polymer is a novel water-soluble zwitterionic copolymer composed of diallylamine hydrochloride salt and maleic acid. This polymer provides us with efficient grafting points for the target element of human blood group A trisaccharide antigen through its carboxyl groups. The characteristics of the polyampholytic polymer are strongly affected by pH levels, which in turn change the sensor properties. Thus, at first we evaluated the sensing properties in terms of signal intensity with various pH levels and concentrations of the polymer solution to achieve good selectivity (Figure 1). We used 5 representative human sera for this optimization, including 4 blood group O and 1 blood group AB. Although the variation of the concentration of anti-A in serum is relatively large even in healthy volunteers, it may due to the difference in individual exposure to the antigen. The serum of blood group AB works as a negative control

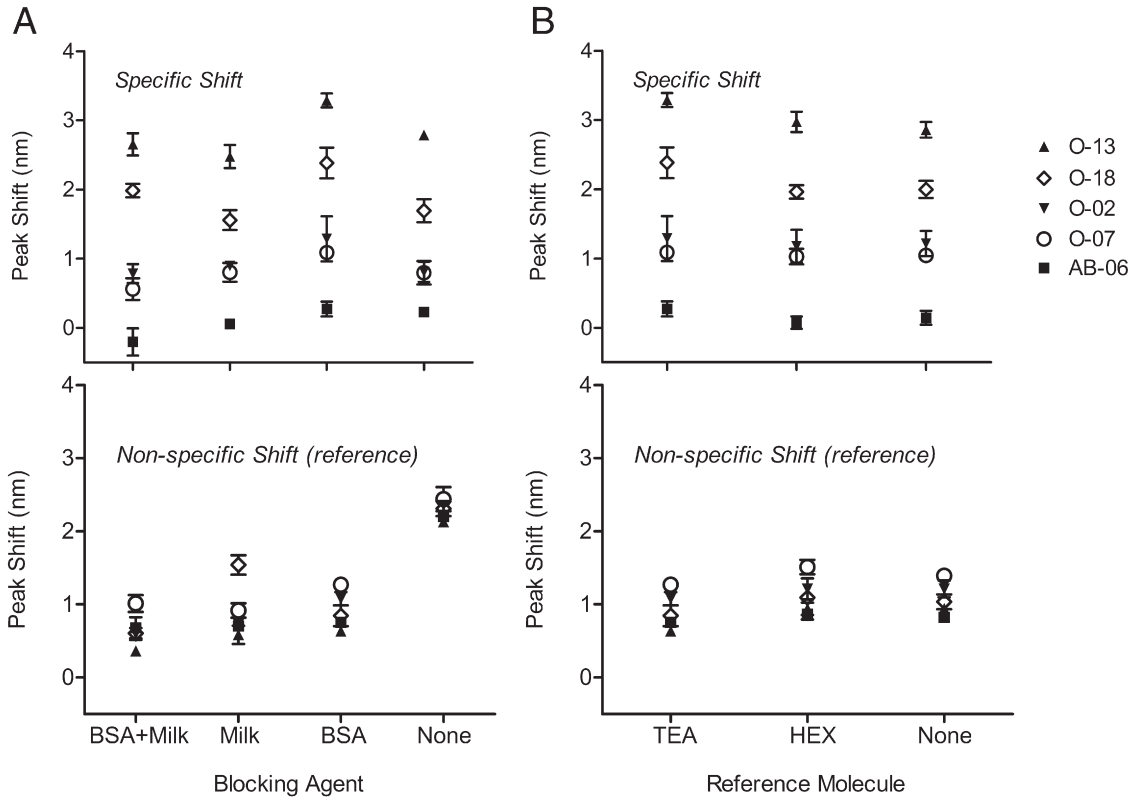

Figure 2. Comparison of effects of blocking agents and reference molecules on sensor responses (shift of λ_{max}). **(A)** Effect of blocking agents: BSA+Skim milk, Skim milk, BSA and None. **(B)** Effect of reference molecules: TEA, HEX and None. The upper graphs represent data from signal wells, whereas the lower graphs represent data from reference wells. Data were acquired using the sera from 4 blood group O and 1 blood group AB volunteers. Error bars represent standard errors.

because we do not expect any response to the blood group A antigen from the serum of blood group AB. We chose the optimum condition for coating as 8.4×10^{-4} % (w/w) at pH 2.0 hereafter, which was in the middle of the stable condition having a large response.

In the second step of the optimization, we further investigated the selection of blocking agents and reference molecules, which would reduce the unknown signals from crude samples and enable a single-step, label-free measurement. We tried well-known blocking agents of BSA, skim milk and their combination. For reference molecules, which were immobilized on the surface of AuNPs in the reference well, we tried TEA and HEX. Based on the result shown in Figure 2, we conclude that choosing BSA and TEA for blocking agent and reference molecule, respectively, can achieve larger signal. Then, we were convinced that the optimum condition of our assay had been achieved and we were ready to proceed to measure various serum samples. Again, we observed a good selectivity towards anti-A antibody also in this optimized condition by using serum of blood group AB.

Validation: Concentration-dependent shifts of λ_{max} by purified human anti-A antibody

We confirmed concentration-dependent shifts of λ_{max} as basic characteristics of our LSPR sensor using purified target antibody: human anti-A antibody (Figure 3). We fitted this profile assuming a 1:1 binding model between anti-A and blood group A antigen. The fitted curve provides us the dissociation constant (K_d) of 1.5×10^{-4} g/mL. Due to the difficulty of preparing a higher concentration of purified anti-A antibody from human blood, we did not measure λ_{max} in a higher concentration range than 40 μg/mL. However, with using the serum that has the highest concentration of anti-A antibody, we observed the similar trend of shifts of λ_{max} across our experimental concentration range.

Challenge: single-step, wash-free measurement of serum samples

We applied our assay to the various samples from volunteers and investigated the applicability of a single-step measurement in crude samples. The difference between the process of "wash" and "wash-free" measurement concerns whether we replace the serum in each well with a buffer solution before performing the spectrum measurement. In the single-step "wash-free" measurement, we do not discard the serum and we proceed directly to the spectrum measurement. The major purpose of this "wash" operation is to significantly reduce the signal from the unknown species in the serum and improve the accuracy of the measurement. Figure 4 compares the signals from both operations. Interestingly, the slope of linear regression between 2 methods is identical to unity with $R^2 = 0.91$. This means that there is no significant difference between them. We therefore conclude that the single-step "wash-free" measurement works

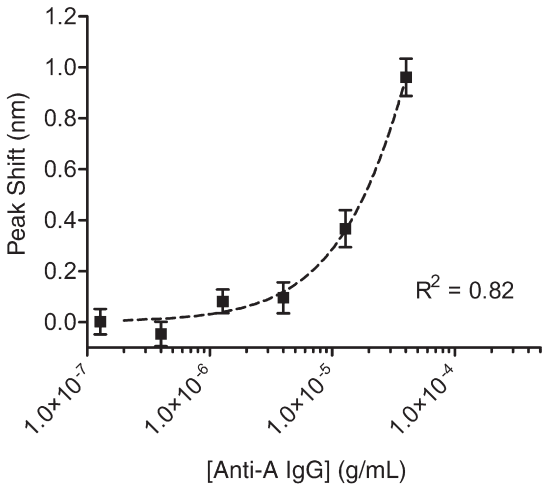

Figure 3. Anti-A concentration-dependent shifts of λ_{max}. Dotted line shows a sigmoidal fitting to the experimental data. Error bars represent standard errors from 8 samples.

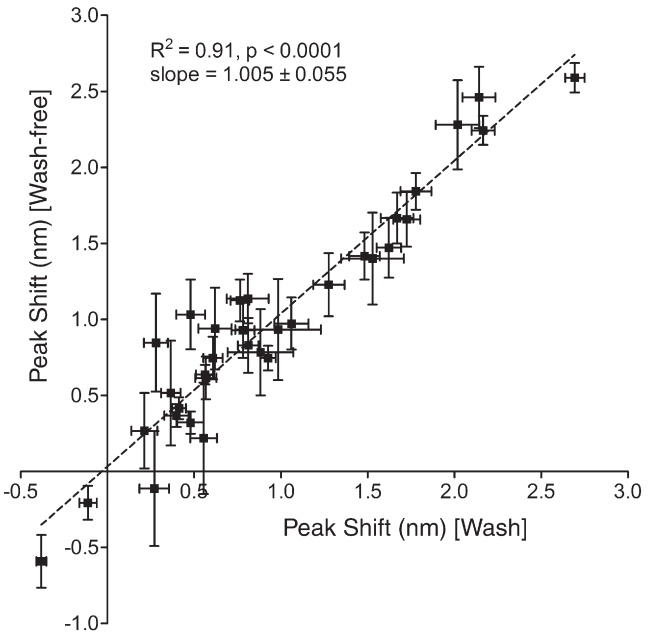

Figure 4. Wash-free measurement. Correlation between "wash-free" and "wash" measurements. Dotted line was determined by linear regression analysis (slope = 1.005, R^2 = 0.91). Error bars represent standard errors.

equivalently to the standard "wash" operation without any significant interference from the serum in the well.

Comparison of the single-step assay with other methods

Finally, we compared our single-step "wash-free" assay with other methods, i.e., TT and SPR, using the common 41 serum samples (Figure 5, *A*). Because two major isotypes of antibodies exist in serum, i.e., IgG and IgM-types, we conducted a supplemental measurement using the secondary antibody (anti-human IgG) that specifically detects the amount of IgG-type anti-A antibody bound on the AuNPs and computed the correlations separately in those two types of measurements (Table 1). As for the TT method, the procedure we used for the computation of correlations is generally assumed to measure IgG-type antibody only. We observed at least moderately strong correlations among the methods. For the IgG-type antibody, the correlation coefficient between LSPR and TT is smaller than that observed between LSPR and SPR (0.66 vs. 0.79). This is possibly due to the less quantitative nature of the TT method, in which results are discrete values. For instance, the TT results of many samples were zero in Figure 5, *B*. On the other hand, correlation coefficients between LSPR and SPR are approximately 0.8 (strong) for both types of measurement, and more than 60% of the variability in the LSPR results is explained statistically by SPR (Figure 5, *A*). In particular, we did not find a difference in the correlations between the measurements of total antibody and IgG-type in the comparison of LSPR and SPR. Thus, we conclude that LSPR and SPR measured the similar types of antibody in our assay because two types of measurements (i.e., total and IgG-type antibody) did not change the correlation between the two methods.

Discussion

Development of a rapid, accurate and sensitive diagnostic method is favored in clinical practices from both financial and ethical points of view. With this goal, many types of new biosensors have been proposed and presented to the clinical community for use in diagnostics. Meanwhile, TT and ELISA have been widely used along with accumulated empirical evidence and increased confidence, even though they require sophisticated operations and substantial time to complete. We believe that one of the tipping points where the clinical community will accept a new method depends on the extent of its simplicity of operation and reproducibility of results relative to the conventional methods.

In this study, we demonstrated a more efficient approach to quantify the amount of antibodies in sera while fulfilling practical requirements. We optimized our assay to detect antibodies in both nano-optical[15] and surface chemical aspects as shown in Figures 1 and 2. The optimal arrangement of immobilized AuNPs and the use of a polyampholytic polymer are two essential elements of our method. The former enhanced the sensitivity and the latter enabled efficient surface chemistry and high selectivity. Though the zwitterionic copolymer looks difficult to control in a stable manner, we found its optimum condition to facilitate surface modification. We believe that larger signal responses using the acidic polymer solution are caused by the dense attachment of the polymer on the surface of AuNPs due to static attraction between positively charged polymer chains and negatively charged AuNPs. The capability of blocking nonspecific binding is similar for BSA and skim milk (Figure 2), but we cannot expect a synergetic effect using both materials simultaneously. From our experience, BSA seems to be a more stable agent for long-term storage. To optimize a reference well, we tried 2 kinds of molecules (i.e., TEA and HEX). As expected, slightly better trends were achieved using more hydrophilic molecules, which have a relatively similar

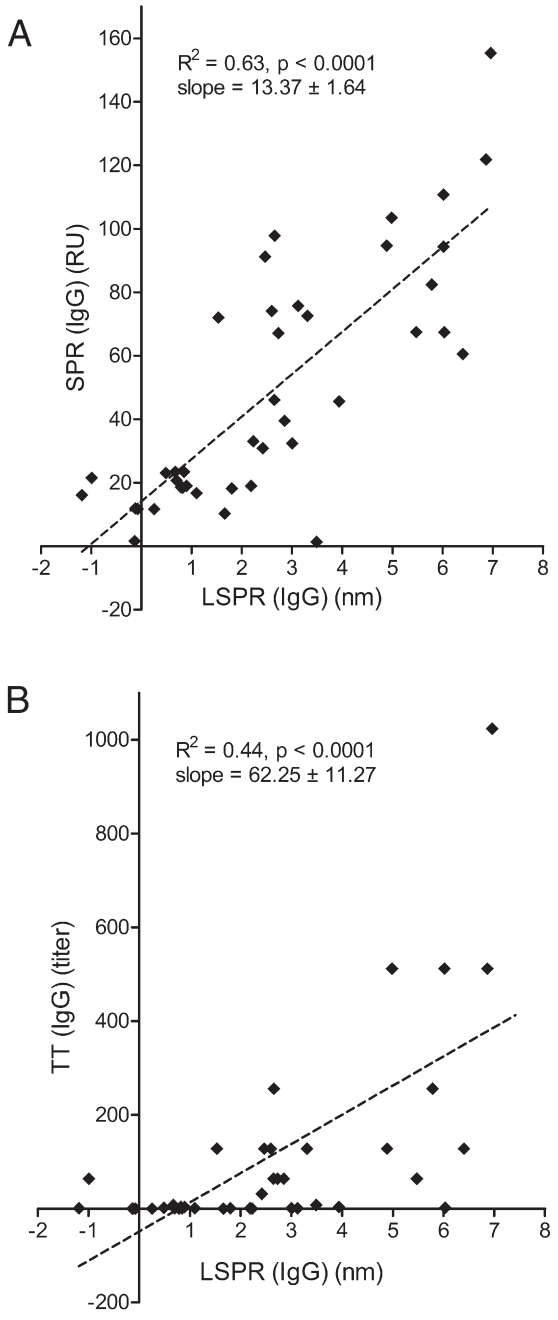

Figure 5. Correlations between TT, SPR and LSPR measurements of 41 volunteers. **(A)** Between SPR (IgG) and LSPR (IgG). Dotted line was determined by linear regression analysis (slope = 13.37, R^2 = 0.63). **(B)** Between TT (IgG) and LSPR (IgG). Dotted line was determined by linear regression analysis (slope = 62.25, R^2 = 0.44).

molecular characteristic to the trisaccharide antigen. Attained sensitivity and selectivity both contributed to the success of single-step "wash-free" measurement. A calibration plot of λ_{max} as a function of anti-A antibody concentration shows a detection limit of <10 μg/mL (Figure 3). This plot is based on the specific shift subtracted by nonspecific shift. Thus, a lower bound of error bar should simply exceed zero at the detection limit. Using a streptavidin-biotin reaction in diluted human blood, Wang et al

Table 1
Pearson correlation coefficients between assays

	TT (IgG)	SPR (Total)	SPR (IgG)
LSPR (Total)	0.70	0.80	0.78
LSPR (IgG)	0.66	0.80	0.79

The correlation coefficients were computed with the data from the common 41serum samples.

obtained a detection limit of about 3 μg/mL.[13] We are convinced that our results are within a reasonable range using an antigen-antibody reaction.

In comparison with other methods, we observed at least moderately strong correlations among them (Table 1). A statistical interpretation of R-squares is that at most, 44% and 63% of variability of the results in LSPR were to be explained by TT and SPR, respectively (Figure 5). In other words, we should be cautious about considering other factors that influence the measurements. This is because such methods do not necessarily measure exactly the same events that LSPR measures. Particularly, TT is less quantitative, for which results are discrete values based on serial dilutions and visual observation. Moreover, SPR uses a flow-cell system, which means it detects a dynamic process of binding events, not a static process. These diferences would contribute to the decreased correlations or R–squares.

It is well known that human serum has several isotypes of antibody dominated by IgG and IgM, which was confirmed by the TT method here. We consider it in the case of our assay. We suppose that LSPR and SPR detect mostly the IgG-type antibody, which we presume from their similar responses even without the use of the secondary antibody. That secondary antibody was used to distinguish IgG and IgM. This is, to some extent, due to the higher K_d of IgM antibodies in a monovalent intereraction[18] between antigen-binding region and immobilized antigen than that of IgG. In our assay by LSPR or SPR, IgG and IgM competed against each other for the same targeting antigen. Our observation of results, however, is that the major responses are caused by the reaction of IgG, owing to its higher affinity to the antigen than IgM. Therefore, we conclude that LSPR could substitute for the function of SPR in measuring antibody titers and improve the operability in clinical practice because of their similar diagnostic characteristics in terms of the isotypes of antibody to be measured.

Two features of our method are worth mentioning with respect to the clinical diagnostics. First, our assay is simple, and a single-step operation is possible. Figure 4 reveals the equivalency of responses between "wash" and "wash-free" measurements. The near-zero intercept also indicates the competence of our technique to prevent nonspecific adsorption from serum contents. This is owing to the spatial characteristic of the LSPR biosensor as illustrated in the Graphical Abstract. Specifically, LSPR has a smaller sensing volume than SPR, which is appropriate to exclusively detect the surface binding events. In addition, we have optimized the sensing volume for detecting antibodies as reported previously.[15] We emphasize this property because none of the conventional methods is able to measure quantitatively with a single step. This observation would bring a huge benefit in clinical practice because the only required

procedure to get accurate diagnostic results is to put the test solution into a reaction well. This means a substantial reduction of total operational time to measure the concentration of antibodies. In addition, it could eliminate other laborious manual steps like washing wells, which can cause deviation in the responses obtained by many laboratory technicians in clinical practice. Furthermore, intermittent monitoring of specific markers in the sample could be easily conducted in a clinical setting using our method because the quality of each measurement well can be precisely controlled. For example, monitoring the change in the amount of anti-human blood group antigen antibodies is essential for prognosis after the ABO-unmatched transplant. The risk of adverse events in the case of ABO-incompatible living-liver transplantation would decrease significantly with timely monitoring and appropriate remedies. With the help of our methods, physicians will have better opportunities to more accurately follow up on patients' critical responses to the transplants than in the past.

Second, as our assay used microtiter plates, we are easily able to multiplex the measurement because the required sample is small and the operation is simple. In many situations, physicians are interested in a set of different antibodies or biomolecules to improve the performance of diagnosis of a patient's condition. For instance, multiplexed markers for autoimmune diseases or tumors are used and explored.[19-22]

Further intensive study is necessary to improve this assay. A major drawback to our demonstrated method is the use of reference wells, which is also a fundamental challenge in the development of label-free optical biosensors. This duplicates the operation to improve signal intensity. However, we hope to find a way to obtain accurate results even without reference wells. Moreover, this single-well measurement will provide a real-time measurement of molecular binding events, which enables us to understand the affinity and kinetics of that binding event similarly by the SPR method.

In conclusion, we have shown a single-step, label-free quantification of anti-A antibodies in human serum. Our method achieved a simple, efficient and reliable way to specifically detect antibodies in crude serum samples. These results point to the vast applicability of our LSPR method to measure the level of antibodies in the test solution by adopting various kinds of antigens immobilized on AuNPs. We believe that the LSPR method will have the potential to be routinely used as a rapid and cost-effective diagnostic method in the future.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.nano.2011.02.002.

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