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Original Article

Clinical application of a novel sliver nanoparticles biosensor based on localized surface plasmon resonance for detecting the microalbuminuria

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In order to explore the clinical application of the nanobiosensor based on localized surface plasmon resonance (LSPR), we used our LSPR biosensor to detect the microalbuminuria in this work. The sliver nanoparticles were fabricated by using nanosphere lithography. The anti-human albumin antibody was immobilized on the sensor surface by amine coupling method. The different concentrations of commercial albumin and albumin in urine samples from three mild preeclampsia patients were determined according to the peak of LSPR extinction spectra. Under optimum conditions, our results showed that the biosensor displayed a detection limit of 1 ng/ml and wide dynamic range of 1 ng/ml to 1 μg/ml. Furthermore, the microalbuminuria of three patients was determined by our biosensor within a short assay time, without sample purification. This biosensor proposed herein is easy to prepare and could be used for low-cost, rapid, label-free, and sensitive screening of the microalbuminuria. This approach provides a promising platform for developing clinical diagnostic applications.

Keywords localized surface plasmon resonance (LSPR); biosensor; microalbuminuria

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Introduction

Along with the development of nanometer science and technology, nanoscale particles show potential applications in many fields due to their unique optical and electrical properties, which attracts greatly the interest of scientists [1–4]. The attractive optical properties of noble metal nanoparticles are connected to their localized surface plasmon resonance (LSPR). The applications of LSPR based on nanoparticles have been a research hot spot in recent years [4–9].

The LSPR is an optical phenomenon generated by collective oscillations of the electron gas in metal nanostructures surrounded by a dielectric environment [10]. The metal nanoparticles possess strong absorption in the UV–visible region [11], often coined as localized surface plasmon absorption. Typical materials for plasmonic applications are noble metals, especially silver or gold. The LSPR sensor of silver presents sharper and more intense LSPR bands than that of gold [10]. Local refractive index changes such as those induced by biomolecular interactions at the surface of nanostructures can be monitored via the LSPR peak shift. The extinction and scattering spectra of the nanoparticle and the peak wavelengths of the LSPR extinction maximum spectra (λ_{max}) depend on the nanoparticle composition, size, shape, orientation and local dielectric environment [12,13]. Just as surface plasmon resonance (SPR), biosensor technique can obtain the affinity information on biomolecular interactions [14], this LSPR biosensor can also translate a biospecific interaction into a detectable signal, which is between a ligand in solution and a binding partner immobilized on biosensor surface. However, this nanobiosensor is rarely applied in the field of clinical medicine by far [10]. The development of its use and commercialization is a future prospect and challenge.

Previously, we have achieved the detection of biotin–streptavidin system using our homebuilt nanobiosensor [15]. In order to explore the clinical application of this biosensor, we used LSPR-based sensor to detect the microalbuminuria of patients.

Albumin is a soluble, globular unglycosylated and monomeric protein, which comprises about one-half of the blood serum protein. In a properly functioning body, the albumin present in urine is abnormal, because it should be retained in bloodstream by kidneys. More than normal amount of albumin in urine indicates that protein is leaking through the vascular membranes of kidneys.

A microalbumin urine test can demonstrate the presence of albumin in urine. Microalbuminuria is an independent risk factor for the high incidence and fatality rate of cardiovascular disease (CVD) in diabetes mellitus (DM) [16,17], which is also one of the biomarkers for the endothelial dysfunction [18]. Screening microalbuminuria could identify the patients that are at high risk for CV events with pregnancy-induced hypertension and DM [19]. The early diagnosis and treatment may delay or even prevent the onset of CVD or complications.

Some testing measures for detecting albuminuria have been developed, which include fluorescence-based immunoassays, radioimmunoassay (RIA), high-performance liquid chromatography (HPLC), sodium dodecyl sulfate electrophoresis and immunonephelometry (INM). Although these methods are quite reliable and highly sensitive [20–22], all of them are time-consuming and labor-intensive protocols. Moreover, the fluorescence-based immunoassays and RIA require labeled molecules, and the RIA method is cumbersome and requires special laboratory facilities. HPLC cannot absolutely differentiate albumin from other urinary proteins unless it is combined with special detection strategies. INM requires sophisticated and expensive equipments and rigidly matched reagents [23]. These various reasons limit their clinical applications. So a simple, rapid, low-cost, label-free and sensitive technique for urinary albumin detection is extremely needed for clinical medicine.

To overcome the disadvantages of those tests mentioned above, in this study, we developed a novel LSPR-based sliver nanoparticles biosensor for detecting the albumin and the patient's urine sample. We hope this clinical research with the nanobiosensor lays the foundation for its further clinical applications.

Materials and Methods

Materials

11-mercaptoundecanoic acid (MUA), *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC), the human albumin, and the mouse anti-human albumin monoclonal antibody were purchased from Sigma (St. Louis, USA). The recombinant human IL-17 was obtained from R&D Systems (Minneapolis, USA). Ag wire was obtained from Jubo Company (Beijing, China). Quartz glass substrates were purchased from Juke Company (Chengdu, China). Polystyrene nanospheres and glass nanospheres were received as a suspension from Water Co. (Milford, USA). Ultrapure water (18.3 MΩ/cm) from Millipore Co. (Boston, USA) was used in all preparations. Other chemicals used in the present study were all of reagent grade.

Preparation of LSPR biosensor

The integrated LSPR sensor used in this study is our home-built system. The preparation of glass substrate and nanoparticle is carried out as described previously [15,24]. The sliver nanoparticles were fabricated using nanosphere lithography (NSL). The peak wavelength (λ_{max}) excited by the Ag nanoparticles was measured and recorded using a UV–visible spectroscopy (Sciencetech 9055) (Sciencetech Corp, Ottawa, Canada) with a photomultiplier detector before, during, and after each incubation step. The detection area of the biosensor was ~2 mm × 2 mm. All the extinction spectra were directly derived by using professional spectrasuite software (Ocean Optics Corp., Dunedin, Florida, USA).

All absorbance spectra were taken from a range of 400–800 nm on the UV-visible spectrometer in atmosphere environment. The white light emerging from the optical fiber bundle was presented on the surface of nanochip. The reflected light was coupled with the detection fiber probe of the optical fiber bundle and analyzed by the UV–visible spectrometer. The absorbance strength change was recorded absolutely according to the concentration of analytes. The maximum extinction of each spectrum was located by calculating its first derivative. A shift toward longer wavelengths was referred to as a red shift, and was denoted as (+); whereas a shift towards shorter wavelengths was referred to as a blue shift, and was denoted as (−). These wavelength shifts were a more reliable indication of chemical changes at the nanoparticle surface than changes in peak intensity [25]. So the relative wavelength shift, $\Delta\lambda_{max}$, was used to monitor the binding of analytes [9].

Immobilization of antibody on nanoparticle layer surface

Figure 1 shows the experimental procedure for immobilization of anti-albumin antibody. The sliver nanoparticle slice was immersed in 1 mM MUA solution (in ethanol) for 18 h to form a self-assembled monolayer (SAM) of MUA on slice surface, then washed with pure ethanol and dried at room temperature. After incubation for 2 h in EDC/NHS solution to ensure that the carboxyl group of the SAM reacted fully with the EDC/NHS at room temperature. After that, the slice was washed thoroughly by ultrapure water and dried. We diluted the anti-albumin antibody in 10 mM phosphate-buffered saline (PBS, pH 7.4) at various concentrations of 1, 10, 50, or 100 μg/ml to determine the optimum. These different concentrations of antibody were spotted onto the modified surface using a micropipettor of a 50 μl volume and incubated at 4°C overnight. Finally, the antibody-immobilized surface was rinsed thoroughly with PBS and dried at room temperature. The antibody-immobilized spot was formed on the chip surface (Fig. 1).

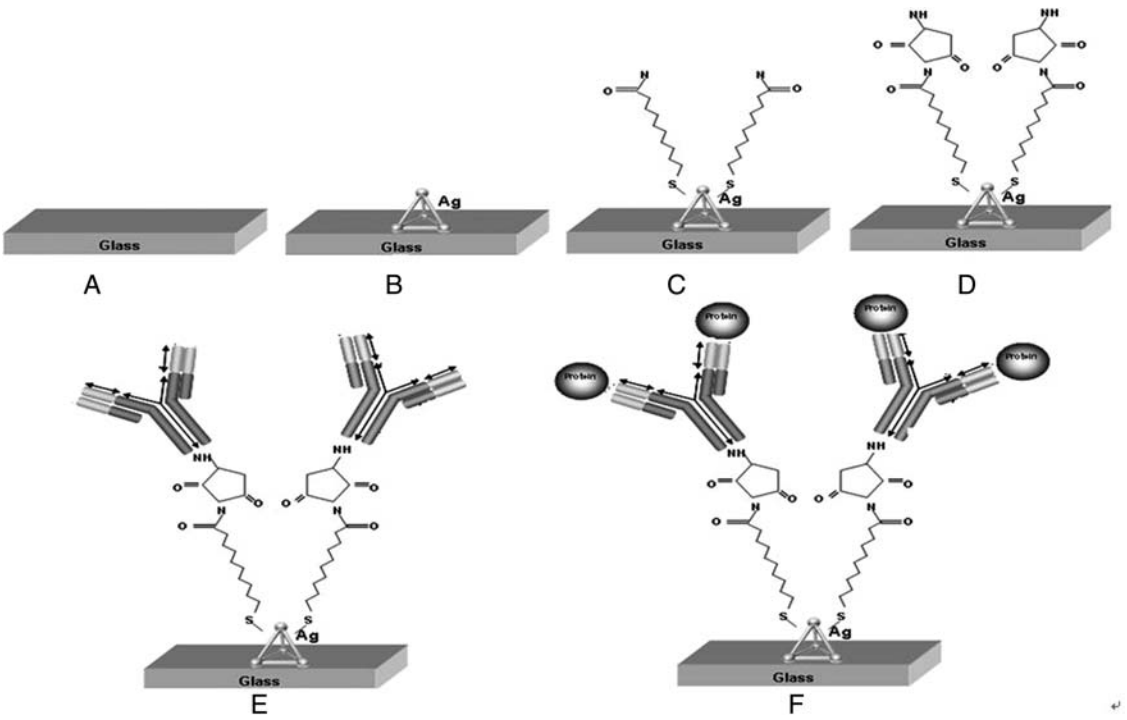

Figure 1 Design of LSPR nanobiosensor for albumin detection (A) Glass substrate. (B) Surface-confined sliver nanoparticles were synthesized using NSL. (C) The nanoparticles were incubated in MUA solution to form a SAM. (D) The substrates were incubated in 75 mM EDC/15 mM NHS. (E) Anti-albumin antibody (10 μg/ml) immobilized the substrate. (F) Varying the concentrations of the albumin or the samples on the substrates completed the albumin immunoassay.

Urine samples of patients

The urine samples were obtained from three mild pre-eclampsia patients, who signed informed consent under an Institutional Review Board-approved protocol at the West China Second University Hospital (Chengdu, China). samples were diluted 1:1000 with PBS before LSPR analysis. Meanwhile, samples were confirmed with the standard clinical chemistry by a Hitachi 7600 analyzer (Hitachi, Tokyo, Japan) in the hospital.

Detection of albumin from human samples using LSPR biosensor

As shown in Fig. 1(F), after the immobilization of antibody on the chips, the commercial albumin solution (1 ng/ml to 1 μg/ml, in 10 mM PBS) and the urine samples were introduced to the nanoparticle layer substrate surface using a micropipettor and incubated at 37°C for 30 min. The incubated chips were rinsed with PBS and ultrapure water, then dried at room temperature. Subsequently, the change of absorption spectrum caused by antibody–antigen reaction was observed.

Nonspecific binding tests

Recombinant human IL-17 which could interact with immobilized anti-albumin antibody was detected on sensor surface, according to the methods mentioned above. The assay was performed in triplicate.

Results

The anti-albumin antibody absorbed on nanoparticle layer surface

The use of the sliver nanochip as a biosensor was investigated using MUA/EDC/NHS and anti-albumin antibody. Briefly, the sliver nanochip was modified with self-assembled monolayer of MUA. An amine-terminated anti-albumin antibody was coupled to the carboxylic acid groups presented by MUA through EDC/NHS activation. Then, the antibody immobilized on chip was investigated by LSPR spectroscopy. Each spectrum was obtained under different surface conditions of deposited sliver nanoparticles. According to Mie's theory, the light extinction maximum and the extinction cross-section of noble metal nanoparticles depend on the refractive index of the surrounding medium [6]. The molecular deposition on the sliver nanoparticles increased the absorbance and red shift of the spectra maximum. Nanoparticles were incubated on SAM for 18 h, and a representative LSPR extinction wavelength was measured to be 577.74 nm [Fig. 2(b)]. These chips were then incubated in the EDC/NHS solution for 2 h, the LSPR wavelength shifted to 582.97 nm [Fig. 2(c)]. After incubation with anti-albumin antibody overnight, the LSPR wavelength shifted to +4.84 nm, giving a λ_{max} of 587.81 nm [Fig. 2(d)]. The optimum concentration of anti-albumin antibody was determined to be 10 μg/ml when the

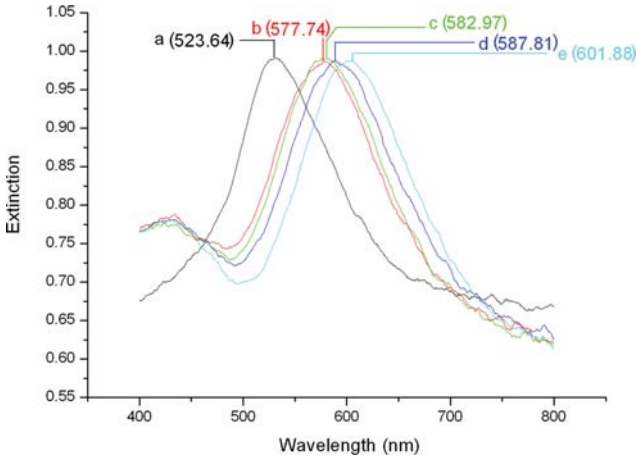

Figure 2 The specific LSPR absorbance peak was observed after each step of preparation of nanobiosensor a, Ag nanoparticles before chemical modification; b, MUA (1 mM); c, EDC/NHS solution; d, antibody (10 μg/ml); e, albumin (1 μg/ml). All spectra were collected in air environment.

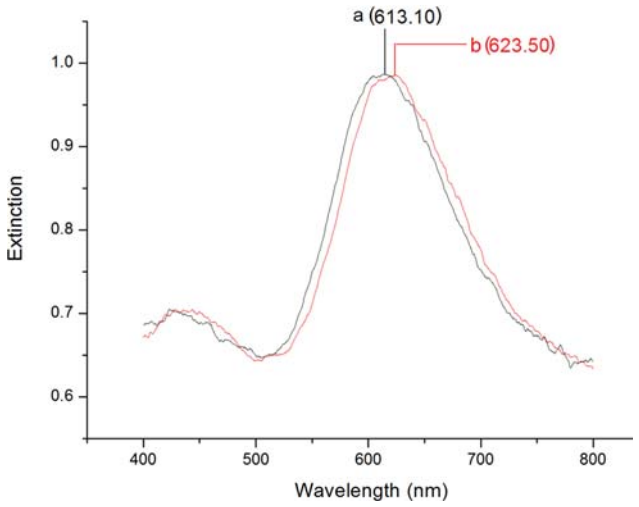

Figure 3 Demonstration of LSPR assay at low concentration a, antibody; b, albumin (10 ng/ml). All spectra were collected in air environment.

maximum absorption peak presented. These distinct evidence showed that the anti-albumin antibody is successfully bound on sliver nanochip surface.

Label-free measurement of albumin

Albumin binding to an immobilized anti-albumin antibody was examined through LSPR spectroscopy. LSPR spectra were measured after chips were incubated with different concentrations of albumin for 30 min and washed as described in the 'Materials and Methods' section. The absorbance strength change was recorded. The extinction wavelength maximum shifted to 5.02 nm (data not shown) with albumin at 1 ng/ml, 10.4 nm (from 613.10 to 623.5 nm) at 10 ng/ml [**Fig. 3(a,b)**]. When the assay was repeated with 1 μg/ml albumin, the maximum shifted to 14.07 nm (from 587.81 to 601.88 nm) [**Fig. 2(d,e)**]. As the concentration of albumin increased to 1.5 μg/ml or decreased to 0.5 ng/ml, no significant changes or blue shift were observed in spectra. So, the biosensor could perform when albumin concentration ranged from 1 ng/ml to 1 μg/ml (**Figs. 2** and **3**).

Detection of the albumin in diluted patients' urine samples

Figure 4 shows the absorbance change of one diluted urine sample. The extinction wavelength shifted to +7.49 nm, from 715.96 nm to 723.45 nm [**Fig. 4(b,c)**]. The other two samples also exhibited remarkable red shift (data not shown). Results indicated that microalbumin exist in the diluted urine samples. The time of testing process was only 40 min. The concentration of albumin in urine samples, which were not diluted, by clinical chemistry test was 8, 9.8, and 9.2 μg/ml, respectively.

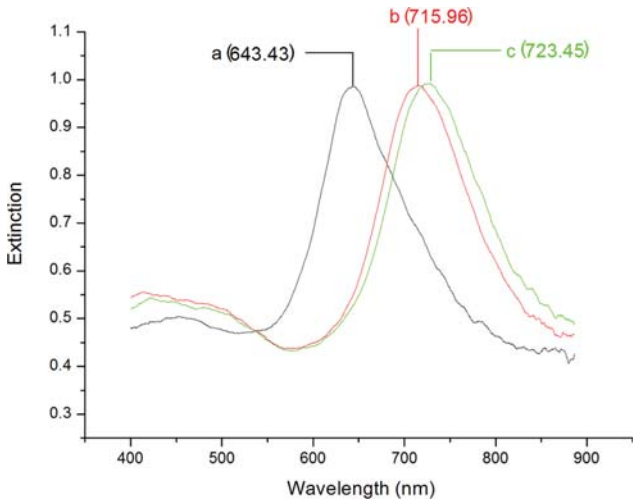

Figure 4 Analysis of one clinical sample of the patient using the LSPR biosensor a, Ag nanoparticles before chemical modification; b, antibody; c, clinical sample. All spectra were collected in air environment.

Nonspecific binding studies

The anti-albumin antibody biosensor surface was exposed to 100 ng/ml IL-17 and yielded a λ_{max} of 714.49 nm [**Fig. 5(a)**], indicating a very small LSPR red shift of +0.73 nm due to nonspecific binding [**Fig. 5(a,b)**]. Little evidence for an LSPR shift could be found within the experimental error ($\Delta\lambda_{max} = 0.61 \pm 0.21$ nm, three trials). Therefore, as expected, nonspecific binding of protein molecules on nanoparticles-covered SAM was found to be quite low.

Discussion

Our results showed that the biosensor displayed a low detection limit of 1 ng/ml and the albumin concentration in

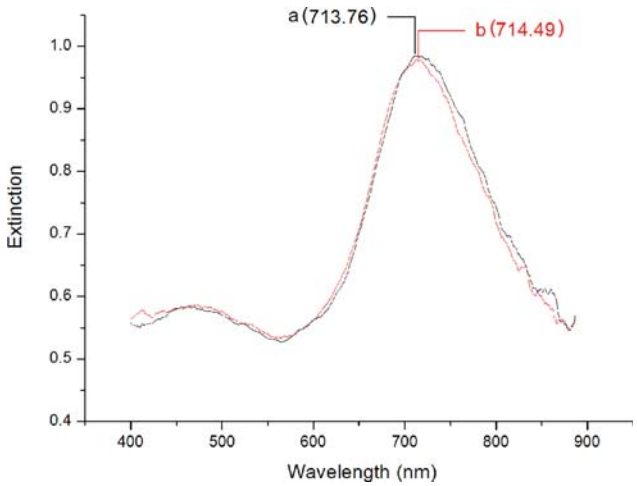

Figure 5 Analysis of nonspecific binding for the LSPR nanosensor a, Ag nanoparticles with anti-albumin antibody; b, IL-17. All spectra were collected in air environment.

the diluted sample could range from 1 ng/ml to 1 μg/ml. our results were in accordance with the data obtained with the standard clinical chemistry method in the hospital. These data suggest a promising future for clinical sample analysis using this LSPR-based biosensor. The very small red shifts in our nonspecific binding studies can be attributed to the removal of a small amount of covalently bound anti-albumin antibody and/or to the slight variation in spectrometer noise, which indicate that no additional mass is detected on the nanoparticle sensor chip.

In addition to the high accuracy and sensitivity, there are many advantages in the LSPR-based biosensor for medical applications. They are as follows: (i) the LSPR biosensor can specifically identify low concentrations of biological substances and potentially be used in a variety of applications including cancer diagnosis, microorganism detection, drug screening, genomics/proteomics research; (ii) this nanosensor is easy to prepare, even by 'nonspecialists' [26] and the operation procedure is more convenient and rapid. No need for purification/separation of the sample greatly shortens the analysis time. The total analysis time, including assembly, incubation, and detection, was $<$40 min. It also can reduce sample volume, which is an important issue in several clinical diagnostic tests for multiplexing purposes; (iii) compared with conventional immunoassay approaches, such as RIA and ELISA, the LSPR biosensor enables a label-free detection of biomolecular interactions and provides more benefits in microalbuminuria detection. This direct assay method avoids complicated procedures involved and the antibodies or proteins retain the native specific property; (iv) compared with the commercial SPR sensor, the LSPR-based biosensor can be implemented using simple, portable, small, and low-cost equipment. The miniaturization and portability of the LSPR sensors are expected to greatly facilitate point-of-care medical diagnostic applications.

The specificity of surface-attached ligands and the passivation of sensor surface can confer high selectivity and specificity for the LSPR biosensor preventing nonspecific binding. As used in ELISA, the surface-attached ligands are established by the combination of highly specific antigen–antibody reaction. The silver nanoparticles are extremely sensitive to small changes in the surrounding dielectric environment. However, the oxidation of liver surface can limit the sensor stability. Due to this reason, the surface of nanoparticles was chemically functionalized, so that they can be used to provide highly sensitive and selective detection of biological targets. The surface of sliver nanochip was modified with a SAM layer which served many functions including stabilizing the nanoparticles, preventing nonspecific binding and immobilizing antibodies to facilitate a protein detection [1].

However, there are still a lot to be improved, such as pushing sensitivity towards single-molecule detection limit and combining LSPR with complementary molecular identification techniques.

This novel LSPR-based biosensor provides an access to a sensitive and rapid detection method with very simple and convenient instrumentation, which will greatly facilitate point-of-service medical diagnostic applications. Especially, the cost for the fabrication of this biosensor, including the cost of optical apparatus, is significantly lower than that of a conventional SPR measurement apparatus. The LSPR biosensor could detect the very low concentration of albumin, which is necessary for the clinical diagnosis. Our future interest will focus on the construction of nanobiosensors for detecting the disease-related biomarkers.

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