A localized surface plasmon resonance based immunosensor for the detection of casein in milk

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Abstract

In this research, a localized surface plasmon resonance (LSPR) immunosensor based on gold-capped nanoparticle substrate for detecting casein, one of the most potent allergens in milk, was developed. The fabrication of the gold-capped nanoparticle substrate involved a surface-modified silica nanoparticle layer (core) on the slide glass substrate between bottom and top gold layers (shell). The absorbance peak of the gold-capped nanoparticle substrate was observed at ~520 nm. In addition, the atomic force microscopy (AFM) images demonstrated that the nanoparticles formed a monolayer on the slide glass. After immobilizing anti-casein antibody on the surface, our device, casein immunosensor, could be applied easily for the detection of casein in the raw milk sample without a difficult pretreatment. Under the optimum conditions, the detection limit of the casein immunosensor was determined as 10 ng/mL. Our device brings several advantages to the existing LSPR-based biosensors with its easy fabrication, simple handling, low-cost, and high sensitivity.

Keywords: Immunosensor; Localized surface plasmon resonance (LSPR); Nanoparticles; Casein

1. Introduction

Immunosensors have a high potential for many applications, because various compounds of interest can be detected at a very high sensitivity [1]. Optical [2–4], piezoelectric [5], and electrochemical [6,7] immunosensors in connection with microfluidic systems [8] have successfully been applied to monitor antigen–antibody binding reactions. In recent years, immunosensors based on surface plasmon resonance (SPR) were developed for the measurement of antigens, which could bind to antibodies immobilized on the biosensor surface [9–11]. Immunosensors based on localized surface plasmon resonance (LSPR) have become strong candidates for the development of miniaturized high-throughput devices [12].

LSPR is a resonance phenomenon of free electron waves in a metal [13]. It occurs in metallic nanostructures, such as nanoparticles, nanotriangles [14], and nanoisland [15]. LSPR is observed when the frequencies of incident photon match the collective oscillations of the conductive electrons of metal nanoparticles. The nanoparticles exhibit unique optical responses within the UV–Vis region [16], where the absorbance shows an exponential decay with decreasing photon energy (the so-called Mie scattering) onto which an LSPR band, specific for the particle material, is superimposed. The frequency and intensity of the surface plasmon absorption bands highly depend on the type of the material (gold, silver, platinum), the size and the shape of nanostructures as well as on their surrounding environment [17–20]. LSPR-based device can be set up without using the specific configurations, for example, the attenuated total reflection (ATR) optical setup or waveguide coupling, it is possible to fabricate very small devices based on the LSPR technique with a simple optical setup.

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With the advances in biosensor technology, many kinds of biosensors based on LSPR have been developed. Using a gold nanoparticle monolayer immobilized on the glass substrate, the increments in the intensity of peak absorbance and the red-shift in the peak wavelength of LSPR band were observed, as the refractive index of the sample solution and the thickness of the coated film increased [21]. Furthermore, the optical biosensor based on the measurement of the transmission absorption spectra has been developed, and the effect of various nanoparticle sizes on the sensitivity of the LSPR-based biosensors was also investigated [22,23]. Other kinds of LSPR biosensors based on the remarkable optical properties of triangular silver nanoparticles and a single silver nanocube were reported [15]. However, the device fabrication has been time-consuming and controlling the uniformity in the size of nanometals has been difficult, and resulted in poor reproducibility. For the improvement of these issues, we developed a gold-capped nanoparticle substrate and achieved the label-free detection of the fibrinogen and anti-fibrinogen antibody reactions, peptide nucleic acid (PNA)–DNA and DNA–DNA hybridization [24,25]. Multi-detection of clinically important proteins was also performed by spotting different antibodies on the surface of our device [12]. In its construction, the silica nanoparticle was used as the “core”, and thin gold films were used as the “shell” coated at the bottom and the top of the “core” (Fig. 1). The device is simple to implement, requiring only a UV–Vis spectrophotometer or a flatbed scanner, and suitable for multiplex analysis with high-throughput monitoring in array-based format.

Today, cow milk allergies represent an important health problem in industrialized countries. It can cause breathing problems, hives and rashes, abdominal pain, and possibly serious weight loss, especially in children [26]. Having a heat stable property, casein occupied 80% and has become the most prevalent allergen in more than 30 types of potentially allergy-causing proteins in milk. The lowest observed adverse effect levels (LOAELs) have been relatively consistent in reports about the milk allergens. Bellioni-Businco determined an LOAEL of 350 mg of protein with children in 1 mL of whole milk [27], and Pastorello found an LOAEL of 0.5 g in freeze-dried milk (~185 mg of protein) with adults [28]. To protect the consumer, the labeling of the milk, when it contains between 0.36 and 3.6 mg of total milk allergens, is mandatory in many countries [29]. Therefore, the development of casein testing kits is an important requirement in allergenic diagnosis.

Several biochemical methods for the detection of whole casein as well as its major fractions in milk have already been reported, such as gel electrophoresis [30], RAST (radio-allergosorbent test) [31], HPLC (high-performance liquid chromatography) [32], SPR [33,34], and ELISA (enzyme-linked immunosorbent assay). Most of these methods require time-consuming sample purification, or separation, and incubation steps. ELISA method, which can detect from 1 ng/mL of the casein in a milk sample has been widely used by researchers. However, the disadvantages of the ELISA include its relatively time-consuming procedure (~4 h) that requires skilled laboratory technicians, as well as specialized laboratory equipment.

In this study, we present an LSPR-based immunosensor for label-free detection of casein in milk as a rapid and user-friendly alternative to conventional techniques. Using protein A in the antibody immobilization procedure, our method is promising for orientating the anti-casein antibody on the sensor surface, thus making this approach become simple, effectively and sensitively [12,24]. The anti-casein antibody was immobilized on the gold-capped nanoparticle substrate surface and the optimization as well as characterization of the casein immunosensor was then performed in the following sections.

2. Materials and methods

2.1. Materials

4,4'-Dithiodibutyric acid (DDA) for self-assembled monolayer (SAM) formation on the gold substrate surface was purchased from Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) for the activation of the carboxyl group of DDA was supplied by Dojindo Laboratories (Kumamoto, Japan). N-hydroxysuccinimide
PA). Slide glass substrate (S-1111, 76 layer were purchased from Polysciences Inc. (Warrington, PA). Slide glass substrate (S-1111, 76 × 26 mm, thickness: 0.8–1.0 mm) was purchased from Matsunami Glass Ind. Ltd. (Osaka, Japan). Electronic grade of sulfonic acid and hydrogen peroxide for cleaning the slide glass substrates were purchased from Kanto Kagaku (Tokyo, Japan). Protein A, Fc binding grade, was purchased from ICN Biomedicals (Asse, Belgium). The blocking solution, ethanol amine, was obtained from Sigma-Aldrich (USA). Casein from bovine milk was purchased from Calbiochem (German). The anti-casein antibody was obtained from Biogenesis (England, UK). Two other milk proteins, α-Lactalbumin and β-Lactoglobulin from bovine milk, were purchased from Sigma-Aldrich (USA). All other reagents were analytical reagent grade, and solutions were prepared with ultrapure water (18.3 MΩcm) obtained from MilliQure.

2.2. Apparatus

For the deposition of gold and chromium layer on the slide glass substrate, a thermal evaporator (SVC-700TM/700-2) was purchased from Sanyu Electron Co., Ltd. (Tokyo, Japan). For monitoring of the base pressure, an analog ionization vacuum gauge was utilized (GI-TL3, ULVAC, Kanagawa, Japan). The growth rate in thickness was monitored using a quartz crystal microbalance (QCM, Model TM-200R, MAXTEK Inc., CA, USA). Spectrophotometer (USB-2000-UV–Vis, wavelength range: 200–1100 nm), tungsten halogen light source (LS-1, wavelength range: 360–2000 nm), and optical fiber probe bundle (R-400-7 UV–Vis, fiber core diameter: 200 μm, wavelength range: 250–800 nm) were purchased from Ocean Optics (Dunedin, USA).

2.3. Fabrication and characterization of the gold-capped nanoparticle substrate

The fabrication process of gold-capped nanoparticle substrate is shown in Fig. 2a. First, the slide glass substrates, after washing, were used to deposit a chromium layer of 5 nm and a gold layer of 30 nm in succession. The thermal evaporator was used at a base pressure of 8 × 10⁻⁶ Torr. The growth rate was monitored using QCM, and manually adjusted to 1.0 Å/s. Then, the formation of SAM was achieved by introducing 1 mM of DDA solution onto the gold-capped nanoparticle substrate surface and incubating for 1 h.

The silica nanoparticles were modified with amino groups on their surface by a reaction with 1% (v/v) γ-APTES solution in ultrapure water for 24 h at room temperature (RT) by stirring continuously [25]. After the surface modification, γ-APTES solution was removed in the centrifugal operation for 1 h at 3500 rpm and the recovered nanoparticles were washed with ultrapure water. Both the washing and centrifugal operations were repeated three times. Then, surface-modified nanoparticles were dried up for 5 min at 120°C. Silica nanoparticles modified with amino groups were thus obtained and stored in a desiccator until use.

After treatment with 400 mM EDC for 1 h, the carboxyl groups of the DDA were activated and thus could form esters with the amino groups of silica nanoparticles. The nanoparticle-attached gold substrates were rinsed thoroughly with ultrapure water to remove the excess surface-modified nanoparticles, and dried at RT. Finally, the top gold layer (30 nm) was deposited on the nanoparticle layer using thermal evaporator.

After these fabrication procedures, the optical characteristic evaluation of the gold-capped nanoparticle substrate was carried out in the absorbance spectra range of 400–800 nm using the UV–Vis spectrometer at RT.

To examine the quality of the substrate surface, AFM was carried out in tapping mode using silicon tips and cantilevers with a nominal spring constant of 18 N/m for scanning in air. All reported images were acquired at scan rates in the range of 0.25–0.50 Hz. The surface coverage of the nanoparticles was calculated using calculation software including AFM operating software.

2.4. Fabrication of the casein immunosensor

The anti-casein antibody immobilization on the gold-capped nanoparticle substrate was carried out in a similar model with the formation of nanoparticle monolayer (Fig. 2b). The SAM formation was achieved by introducing 1 mM of DDA solution onto the gold-capped nanoparticle substrate surface for 1 h. SAM functionalization was carried out with 400 mM EDC for 1 h, and then 100 mM NHS solution added to the SAM-functionalized surface for 1 h. Protein A solution was introduced subsequently on the surface for 1 h. The anti-casein antibodies were immobilized onto the protein A-modified surfaces for 1 h, which reacted with the Fc region of the IgG antibodies. Then 1 M ethanol amine was introduced on the surface as the blocking solution. Finally, the antibody-immobilized surface was rinsed thoroughly with 20 mM phosphate buffered saline (PBS, pH 7.4) and dried at RT.

2.5. Label-free detection of casein using casein immunosensor

The sample solution of 20 μL was introduced onto the antibody-immobilized surface for 30 min. After the incubation step for antigen–antibody reaction, immunosensor was washed using PBS with 1% (v/v) Tween-20 to suppress the nonspecific adsorption. The detection of casein concentrations could be performed by monitoring the peak
absorbance intensity increments using UV–Vis spectrophotometer at RT. All absorbance spectra were carried out from 400 to 800 nm. For the evaluation of the specificity of these casein immunosensors, two other allergenic proteins in milk, α-lactalbumin and β-lactoglobulin, were exposed to the casein immunosensor surface.

In the pretreatment of the milk sample, the top fat layer was removed by centrifugation (1800g, 4 °C, 15 min), following the determination of the casein concentration in milk using an ELISA kit from Nippoham Co., Ltd (Osaka, Japan) with the detection limit of 1 ng/mL. After these treatments, the casein concentrations in milk were measured with the same procedure that was described above.

To determine the validation of our method, a desired amount of exogenous pure casein was spiked into the raw milk samples. A series of dilutions were prepared (0.1–10 μg/mL) and measured using our casein immunosensor. The recovery of concentrations was calculated.

3. Results and discussion

3.1. Characterization of the gold-capped nanoparticle substrate

The optical characteristics of the LSPR-based sensor using noble metal nanoparticles were reported with the red-shift in the peak wavelength [22,35] and the increment of

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![Diagram of LSPR-based biosensor](image_url)
the absorbance intensity [21,24,36] when the thickness of biomolecular layer increased and the refractive index changed due to the reaction between the solution under study and the receptor immobilized on the surface. In our gold-capped nanoparticle substrate, we also observed a significant increase in the absorbance intensity of the LSPR.

Fig. 3. LSPR signal and AFM image of the gold-capped nanoparticle substrate: (a) peak absorbance intensity of gold-capped nanoparticle substrate; (b) AFM image of gold-capped nanoparticle substrate surface.
The absorbance peak of the gold-capped nanoparticle substrate was observed at \( \lambda_{\text{peak}} \approx 520 \text{ nm} \) (Fig. 3a). The optical characteristics of the gold-capped nanoparticle substrate were easy to observe with good reproducibility. Furthermore, the gold-capped nanoparticle substrate surface was analyzed using AFM. The AFM images clearly showed that the nanoparticles formed a monolayer on the glass substrate (Fig. 3b). The aggregation of nanoparticles or the formation of multilayers was not observed. Our results were in agreement with the well-known properties that provided a suitable platform for LSPR-based substrates as reported previously [37,38].

The optical characteristics of the gold-capped nanoparticle substrate, the change in the LSPR absorbance peak, were greatly influenced by the thickness of the biomolecular layers on its surface. The absorbance strength increments caused by the formation of several biomolecular layers on the surface were observed for NHS, protein A, and anti-casein antibody layers (Fig. 4).

### 3.2. Characterization of casein immunosensor

For the optimization of anti-casein antibody concentrations, the absorbance intensity increments of LSPR signals were measured in a series of anti-casein antibody dilutions (3, 5, 10, 20, 40, and 60 \( \mu \text{g/mL} \)) using 100 \( \mu \text{g/mL} \) of target casein. As shown in Fig. 5, the absorbance strength increments in the LSPR signal caused by the casein and anti-casein antibody reactions were observed. Using protein A concentration of 5 \( \mu \text{g/mL} \), the maximum change was recorded with the concentration of antibody at 5 \( \mu \text{g/mL} \). From these observations, 5 \( \mu \text{g/mL} \) of anti-casein antibody was adopted as the optimum condition to evaluate the casein concentration in the sample solutions.

### 3.3. Performance of casein immunosensor

The determination of casein in PBS was performed with our casein immunosensors. Subsequently, the peak absorbance increments caused by the specific antigen–antibody reactions could be observed (Fig. 6a). The analytical range and sensitivity of the casein immunosensor were investigated by measuring in different concentrations of casein between 0 and 100 \( \mu \text{g/mL} \). As a result, the dependences of absorbance intensity increments on the casein concentrations in milk were established as shown in Fig. 6b. The high accuracy of our assay, which was estimated by exogenous addition of known casein amounts to the raw milk samples, was confirmed by the results obtained from ELISA (Table 1). Under the optimal conditions, a detection limit of 10 ng/mL for casein was calculated. Using our casein immunosensors, the linear range from 0.1 to 10 \( \mu \text{g/mL} \) was achieved.

Furthermore, two other major allergenic proteins in milk (\( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin) had a negligible interference effect on the antibodies immobilized at our devices. When an excess amount of 100 \( \mu \text{g/mL} \) \( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin were exposed to the casein immunosensor, no changes in the absorbance were observed in both cases (Fig. 6c). These results indicated that the stringent washing and the blocking solution treatment as described in Section 2.4 and 2.5 were enough for suppressing the nonspecific reactions in casein immunosensor.

With the high precision, our method using casein immunosensor based on gold-capped nanoparticle substrate is a promising candidate for low-cost and highly sensitive quantification of analytes in a simple and rapid format.
4. Conclusions

In this research, LSPR-based immunosensor based on gold-capped nanoparticle substrate was developed and applied to detect casein allergen in raw milk samples. Our device is easy to fabricate and using the simple optical system with the estimated cost of the total screening procedure is convenient and remarkably lower than that of the conventional SPR or ELISA systems. We anticipate that this technology will be developed and become a new

Table 1
Comparison of the test results obtained from ELISA and LSPR-based immunosensor for the detection of the spiked casein in raw milk samples

| Spiked casein in milk (mg/mL) | Detected casein by ELISA (mg/mL) | Detected casein by LSPR (mg/mL) | Recovery (%) |
|-----------------------------|----------------------------------|-------------------------------|--------------|
| 0.1                         | 5.93 ± 0.11                      | 5.30 ± 0.19                   | 89.4         |
| 0.5                         | 6.44 ± 0.47                      | 6.36 ± 0.21                   | 98.8         |
| 2                           | 7.79 ± 0.58                      | 7.74 ± 0.24                   | 99.3         |

Fig. 6. (a) LSPR responses monitored using (dashed line) bare gold-capped nanoparticle substrate, (dotted line) anti-casein antibody immobilized substrate, (solid line) after the antigen–antibody reactions with 100 μg/mL casein; (b) calibration plot using the milk samples spiked with casein at various concentrations; (c) LSPR responses monitored using (dashed line) bare gold-capped nanoparticle substrate, (dotted line) anti-casein antibody immobilized substrate, (solid line) after the antigen–antibody reactions with 100 μg/mL β-lactoglobulin.
rapid test kit for screening milk allergens with user-friendly operation. LSPR-based biosensor is a promising candidate to create a massively parallel detection capability for food control in a highly miniaturized package.

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