

Detection of Phosphopeptides by Localized Surface Plasma Resonance of Titania-Coated Gold Nanoparticles Immobilized on Glass Substrates

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We herein demonstrate a new sensing method for phosphopeptides by localized surface plasmon resonance (LSPR) using titania-coated gold nanoparticles immobilized on the surface of a glass slide as the sensing substrate and using UV–visible spectrophotometry as the detection tool. Titania has been known to be an effective substrate for binding with phosphorylated species. The detection principle is the shift of wavelength of optical absorption due to SPR of the gold nanoparticles induced by binding of phosphorylated species with titania on the surface of the gold nanoparticles. The feasibility of the approach is demonstrated by detection of tryptic digest products of β -casein and milk. Gold nanoparticles coated with thin films of titania, immobilized on a glass slide, can selectively bind traces of phosphopeptides from complex samples, resulting in a wavelength shift of the absorption band in the SPR spectrum with good reproducibility. The LSPR results are confirmed by matrix-assisted laser desorption/ionization mass spectrometry. The detection limit for the tryptic digest product of β -casein is 50 nM.

Surface plasmon resonance (SPR) arises from collective oscillations of free electrons at metallic surfaces. The binding of specific molecules onto the surfaces of metallic films or nanoparticles can cause a change in the SPR. Thus, SPR methods have been used widely in sensing biomolecular interactions with the surfaces of metallic substrates.^{1–4} Generally, a reflectometry measurement is used for SPR analysis. Absorption spectroscopy is an alternative detection method that was first demonstrated by Okamoto et al.⁵ They observed that a red shift of the SPR absorption band was obtained due to a thin film of polymer binding on the gold nanoparticles immobilized on a glass substrate.⁵ Nath and Chilkoti further explored the uses of absorption spectroscopy to investigate the change of optical properties due to biomolecular interactions on gold nanoparticles immobilized on glass substrates.^{6–8} This

approach is called localized SPR (LSPR) or nano-SPR. A chemisorbed monolayer of gold nanoparticles on glass substrates can be used to transduce ligand–receptor binding at a surface into an extinction change with a sensitivity that is useful for biosensor applications.

In a recent study, Inamori et al.⁹ have demonstrated the feasibility of the conventional SPR technique for the detection of the presence of phosphopeptides from a peptide array. A novel biotinylated zinc(II) chelate compound was generated for recognition of phosphate molecules on the peptide array. Biotin–streptavidin interactions were then detected using a SPR imaging technique. Titania beads have been employed to purify phosphorylated species such as phosphopeptides,^{10–17} which coordinate with titanium on the surfaces of titania substrates through monodentate, bidentate, and bidentate bridging phosphate coordination.^{18,19} We have previously demonstrated that magnetic nanoparticles coated with titania can effectively trap phosphopeptides from complex samples.¹⁷ Thus, we proposed that gold nanoparticles coated with titania (Au@TiO₂) would be capable of selectively trapping phosphorylated species. A red shift in the absorption band in the LSPR spectrum would result from binding of phosphopeptides onto the surfaces of Au@TiO₂ nanoparticles on a glass substrate. The preparation of glass chips for sensing phosphopeptides is quite straightforward without requiring specially designed molecules as capture probes for phosphopeptides. Phosphopeptides in complex samples can be easily determined by LSPR using glass slides immobilized with Au@TiO₂ nanoparticles as the sensing substrates.

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We also confirmed that the LSPR responses are due to the binding of phosphopeptides on the Au@TiO₂ nanoparticles immobilized substrate. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) was employed to validate the sensing results. The combination of SPR and MALDI MS has been reported previously.^{20–29} Nelson and Nedelkov have demonstrated a number of applications for various analytes using the SPR-MALDI MS combination technique.^{20–26} The compatibility of LSPR with MALDI is especially good because the optical sensing substrate can be easily adapted for MALDI MS analysis after SPR detection. Deposition of MALDI matrix on the sensing substrate is the only required step prior to MALDI MS analysis. Thus, the trapped species by the Au@TiO₂ nanoparticles immobilized sensing substrate can be easily characterized by MALDI MS. We initially used the tryptic digest product of standard proteins as the sample to demonstrate the feasibility of this approach. To simulate a real complex condition, the tryptic digest product of milk was used as the sample to further reveal the practicability of this LSPR sensing method for detection of phosphopeptides.

EXPERIMENTAL SECTION

Reagents and Materials. Trisodium citrate, ammonium hydrogencarbonate, sulfuric acid, and hydrogen peroxide were obtained from Riedel-de Haën (Seelze, Germany), while *N*-[3-(trimethoxysilyl)propyl]ethylenediamine (TMSPED) was purchased from Aldrich (Milwaukee, WI). Methanol was obtained from Tedia (Fairfield, OH). Trifluoroacetic acid and acetonitrile were purchased from Merck (Darmstadt, Germany). Bradykinin, 2,5-dihydroxybenzoic acid, insulin, and trypsin were obtained from Sigma (St. Louis, MO). Tetrachloroaurate was obtained from Showa (Tokyo, Japan). Glass slides (18 mm × 18 mm × 0.15 mm) were purchased from Matsunami Glass. Nonfat milk was purchased from a local grocery store.

Fabrication of SPR Glass Slides. Gold nanoparticles were prepared using the Frens method.³⁰ An aqueous tetrachloroaurate solution (0.117 mg/mL, 50 mL) was heated to boiling, and trisodium citrate (1%, 1 mL) was added to the solution while stirring. The color change of the solution from blue to brilliant red indicated the formation of monodisperse spherical particles. The solution was heated continuously with stirring for an additional 5 min after the color of the solution had changed to red. The suspension of gold nanoparticles was centrifuged at 5000 rpm, and the supernatant was used for the following experiment.

Glass slides were pretreated by soaking in a piranha solution [3: 1 (v/v) H₂SO₄/H₂O₂] for 30 min to remove impurities, and then they were washed with water and methanol.

The glass slides were stored in methanol before use. The piranha solution is highly reactive. It should be handled with care. The surface of the glass slide was modified with a thin film of TMSPED by soaking in 0.5 mL of TMSPED dissolved in deionized water (0.275% (v/v)) for 10 min. The slide was rinsed with deionized water to remove unbound TMSPED, dried with a hair-dryer, and heated in an oven at 120 °C for 30 min. After cooling to room temperature, the surface of the slide was coated with the gold nanoparticle suspension (0.5 mL). After standing at ambient temperature for 12 h, the slide was rinsed with deionized water to remove unbound gold nanoparticles and dried using a hair-dryer. Titania sol was prepared by dissolving titanium isopropoxide (2 μL) in ethanol (10 mL). The sol (200 μL) was coated onto the surface of the gold nanoparticles immobilized on glass chips using a spin coater at a speed of 1000 rpm for 15 s followed by a speed of 1300 rpm for 10 s. The slide was then annealed in an oven at 60 °C for 1 h. After cooling to room temperature, the slide was rinsed with ethanol and water and dried using a hair-dryer.

Preparation of Tryptic Digest Products of Proteins. Tryptic digest products of β-casein, cytochrome *c*, and milk were used as the samples. Proteins and trypsin were prepared in aqueous ammonium bicarbonate solutions (50 mM, pH 8.0). β-Casein (or cytochrome *c*) and trypsin, at a weight ratio of 50:1, were incubated at 38 °C for 24 h. The tryptic digest product was used as the sample for LSPR and MALDI MS analysis.

Nonfat milk (0.25 mL) was mixed with the denaturing solution containing urea (8 M) in 50 mM ammonium bicarbonate (pH 8) and incubated in a dry bath at 38 °C for 30 min. Dithiothreitol (90 mM, 50 μL) prepared in ammonium bicarbonate solution (50 mM) was then added to the mixture followed by incubation at 55 °C for 1 h. After cooling to the room temperature, iodoacetic acid (200 mM, 50 μL) dissolved in ammonium bicarbonate (50 mM) was added to the mixture in an ice bath. The sample vial was wrapped with aluminum foil and vortex-mixed at room temperature. After 2.5 h, the mixture was mixed with trypsin (1 mg/mL, 10 μL) dissolved in ammonium bicarbonate and incubated at 38 °C for 16 h. The reaction product was diluted 1000-fold by 0.15% trifluoroacetic acid (TFA) before analysis.

Analysis of Phosphopeptides by LSPR and MALDI MS. The tryptic digest product obtained above (0.5 mL) was applied on the surface of the LSPR substrate obtained above. After incubation for 1 h, the slide was rinsed with the mixture of 0.15% TFA/acetonitrile and deionized water (1:4, v/v) to remove unbound species. After drying with a hair-dryer, the slide underwent absorption spectroscopy and MALDI mass spectrometry. 2,5-Dihydroxybenzoic acid (DHB; 30 mg/mL, 0.4 μL) dissolved in the solution of acetonitrile and water (1:2, v/v) containing 1% phosphoric acid was used as the MALDI matrix. Bradykinin and insulin were used as the internal standards for mass calibrations.

Instrumentation. All mass spectra were obtained using a Bruker Daltonics Biflex III time-of-flight mass spectrometer (Leipzig, Germany) equipped with a 337-nm nitrogen laser, a 1.25-m flight tube, and a sample target having the capacity to load 384 samples simultaneously. The accelerating voltage was set at

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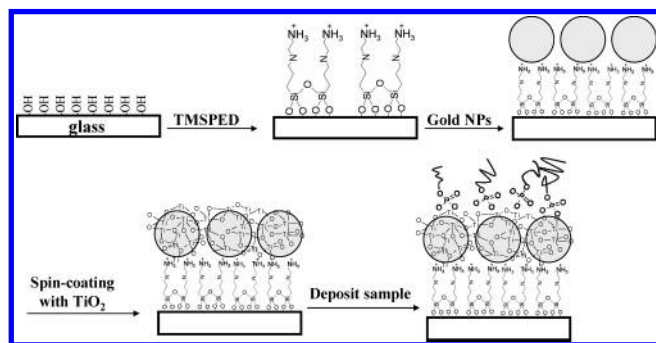


Figure 1. Steps for the fabrication of the LSPR sensing substrate.

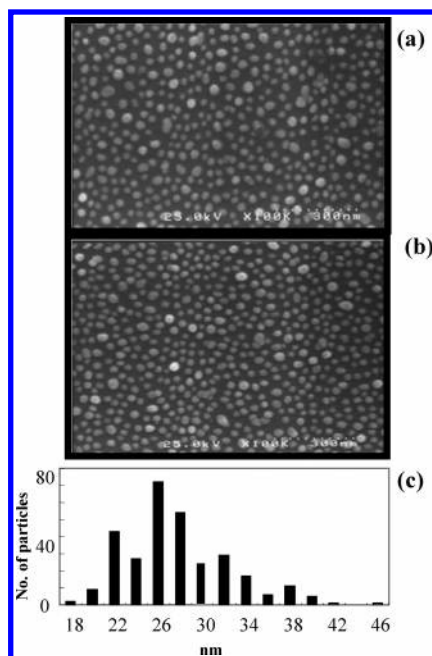


Figure 2. SEM images of a glass slide coated with (a) gold nanoparticles and (b) Au@TiO₂ nanoparticles. (c) The average size of the gold nanoparticles is 27.7 ± 4.8 nm.

19 kV. The absorption spectra were obtained using a Varian Cary 50 spectrophotometer (Melbourne, Australia).

RESULTS AND DISCUSSION

Figure 1 presents the steps of the fabrication of the sensing substrate. Electrostatic attractions were employed to immobilize gold nanoparticles with negative charged functionalities onto the surface of the glass slide, whose surface has been modified by an amine-terminal silane (TMSEPD). After immobilization of gold nanoparticles, the glass slide was spin-coated with a thin film of titania. SEM images of the glass slide coated with gold nanoparticles without (Figure 2a) and with (Figure 2b) the titania coating (Au@TiO₂ nanoparticles) are similar since the thin film of titania is transparent. The size distribution of the gold particles (Figure 2c) on the glass slide was asymmetric with an average diameter of 27.7 ± 4.8 nm. The larger particles were removed by centrifugation at 5000 rpm to yield a more homogeneous distribution with a slightly reduced average size (i.d. 26.6 ± 3.8 nm) prepared from the supernatant after centrifugation (results not shown). It was estimated that the coverage of gold nanoparticles on the glass slide is 34% (~618 nanoparticles/μm²). Nath and Chilkoti⁷ have demonstrated that ~39-nm gold nanoparticles as

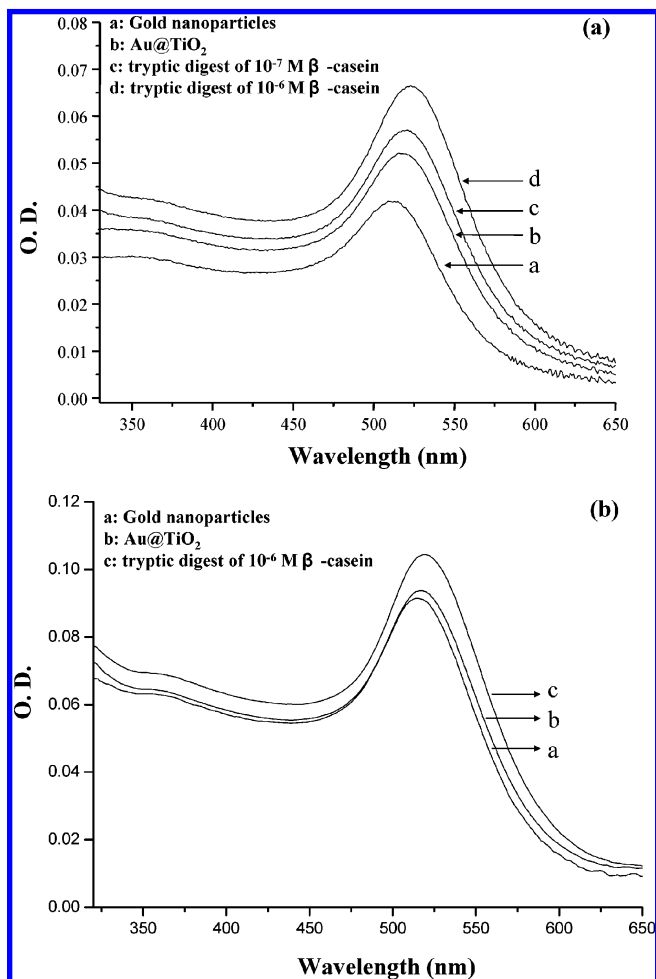


Figure 3. LSPR absorption spectra of the glass slides with and without bound target species. (a) Slides (a) coated with gold nanoparticles (~26 nm); (b) coated with Au@TiO₂ nanoparticles; (c) as (b), but exposed to the tryptic digest of 10⁻⁷ M β-casein; (d) as (b), but exposed to tryptic digest of 10⁻⁶ M β-casein. (b) Slides (a) coated with gold nanoparticles (~39 nm); (b) coated with Au@TiO₂ nanoparticles; (c) as (b), but exposed to the tryptic digest of 10⁻⁶ M β-casein.

sensing particles can obtain the optimum results in their LSPR experiments. Although we used ~26-nm gold nanoparticles for fabricating the substrate for the following LSPR studies, we also displayed the results by alternatively using ~39-nm gold nanoparticles as sensing particles for comparison.

The LSPR absorption spectrum of gold nanoparticles (~26 nm) immobilized on the glass slides shown in Figure 3a (band a) has a maximum absorption at 513 nm. This is red-shifted to 516 nm when the surfaces have been previously coated with titania (band b). After selectively binding target species from the tryptic digest product of β-casein at 10⁻⁷ M, there is a ~3-nm red shift (band c) of the wavelength of maximum absorption relative to band b, before the deposition of the sample. This increased to 5 nm with a 10-fold higher concentration (10⁻⁶ M) (band d). It is also noticeable that the maximum absorption intensity is increased with the concentration of tryptic digest product of β-casein. The LSPR absorption spectrum of gold nanoparticles (~39 nm) immobilized on the glass slides is shown in Figure 3b. It was estimated that the coverage of gold nanoparticles (~39 nm) on the glass slide is ~19% (~150 nanoparticles/μm²) after incubation with a gold

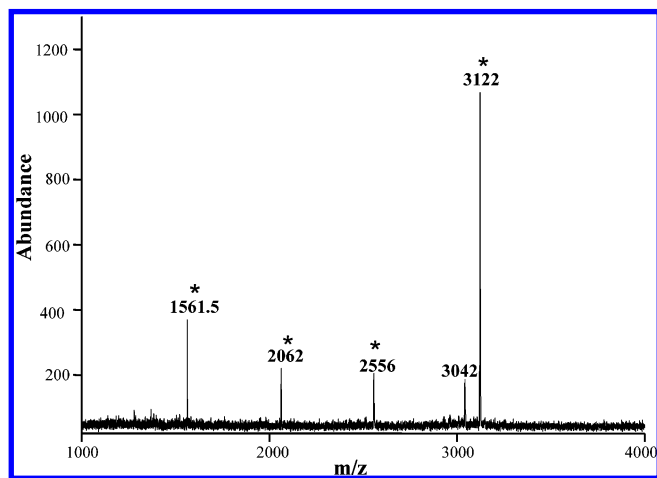


Figure 4. MALDI mass spectrum of the sensing substrate used to obtain absorption band c in Figure 3a. 2,5-DHB was added onto the substrate before MALDI-MS analysis.

nanoparticle (~ 39 nm) solution for 12 h. We tried to increase the coverage of gold nanoparticles on the glass slide; however, the coverage of gold nanoparticles on the slides still remained $\sim 19\%$ after the glass slide was incubated with the gold nanoparticle (~ 39 nm) solution for 18 h. The results we present herein were obtained using $\sim 19\%$ coverage of gold nanoparticle of the glass slide as the sensing substrate. Figure 3b (band a) has a maximum absorption at 515 nm. This is red-shifted to 518 nm when the surfaces have been previously coated with titania (band b). After selectively binding target species from the tryptic digest product of β -casein at 10^{-6} M, there is only a ~ 2 nm red shift (band c) of the wavelength of maximum absorption relative to band b, before the deposition of the sample. The intensity change (I_a) is ~ 0.011 , which is similar to that obtained in Figure 3a ($I_a \sim 0.012$), before and after the deposition of the sample (tryptic digest of 10^{-6} M β -casein). The results indicate that alternatively using gold nanoparticles with diameters of ~ 39 nm as the sensing particles in our experimental condition does not result in any improvement in terms of either wavelength shift or intensity enhancement in our approach. One of the reasons is the LSPR sensing slides we generated are different from that used in the previous study.⁷ Furthermore, the coverage of gold nanoparticles with diameters of ~ 26 and ~ 39 nm on the glass slides is different.

To confirm that the red shift of the SPR peaks is due to the binding of phosphopeptides on the sensing substrate, we employed MALDI MS for characterization. After examination by UV-visible spectrophotometry, the glass substrate used for obtaining the absorption band c in Figure 3a was deposited with MALDI matrix for MALDI MS analysis. Figure 4 displays the MALDI mass spectrum of the sensing substrate used to obtain absorption band c. That is, the tryptic digest product of β -casein (10^{-7} M) was used as the sample for the enrichment of phosphopeptides using the Au@TiO₂ nanoparticles. The peaks marked with asterisks at m/z 2062 (48–63), 2556 (48–67), and 3122 (16–40) correspond to the phosphopeptide residues derived from β -casein. The peak at m/z 3042 corresponds to the loss of a phosphate group from the peak at m/z 3122. The ion at m/z 1561.5 is presumably the doubly charged ion at m/z 3122 because these two peaks always appear simultaneously. It is clear that only the phosphopeptide residues appear in the MALDI mass spectrum. The results demonstrated

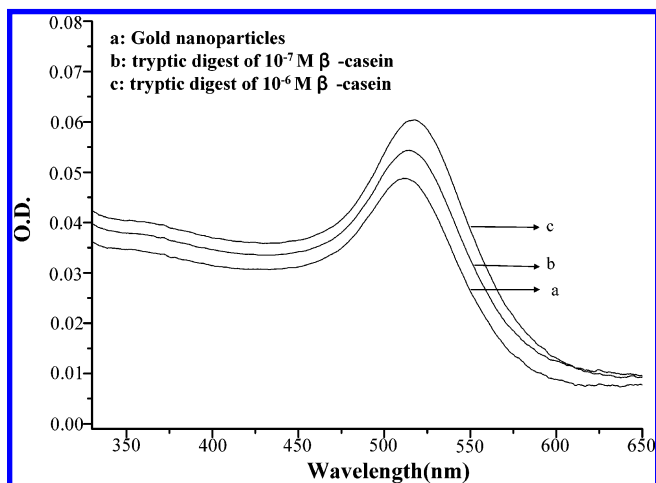


Figure 5. LSPR absorption spectra with and without bound target species using the bare gold nanoparticles (~ 26 nm) immobilized glass slide as the sensing substrate. Slides (a) coated with gold nanoparticles; (b) as (a), but exposed to the tryptic digest of 10^{-7} M β -casein; (c) as (a), but exposed to tryptic digest of 10^{-6} M β -casein.

that phosphopeptides in complex samples can be specifically trapped by our LSPR sensing substrate, to produce a red shift in the LSPR spectra.

Control spectra of the β -casein solution obtained using bare gold nanoparticles as the sensing particles were also carried out. The LSPR absorption spectrum of gold nanoparticles immobilized on the glass slide displayed in Figure 5 (band a) has a maximum absorption at 513 nm. This is red-shifted to 515 nm after selectively binding target species from the tryptic digest product of β -casein at 10^{-7} M. There is a ~ 2 nm red shift (band b) of the wavelength of maximum absorption relative to band a, before the deposition of the sample. This increased to 5 nm with a 10-fold higher concentration (10^{-6} M) (band c). To confirm that the red shift of the SPR peaks is due to the nonspecific binding on the sensing substrate, we employed MALDI MS for characterization. After examination by UV-visible spectrophotometry, the glass substrate used to obtain the absorption band b in Figure 5 was deposited with MALDI matrix for MALDI MS analysis. Figure 6 displays the MALDI mass spectrum of the sensing substrate used to obtain absorption band c. Several phosphopeptides appear in the mass spectrum. The peaks marked with asterisks at m/z 2062 (48–63), 2556 (48–67), and 3122 (16–40) correspond to the phosphopeptide residues derived from β -casein. In addition to these phosphopeptide peaks, there are several peaks at m/z 1384 (206–217) and 1139 (113–122) derived from β -casein revealing in the same mass spectrum. Furthermore, the peaks at m/z 1396 (α -S1/182–194), 1271 (α -S1/169–179), and 1033 (α -S2/174–181) derived from α -casein appear in the same spectrum. It is not surprising to observe the peptide residues derived from α -casein since we used the commercial sample of β -casein with only 90% purity. Previous studies^{31,32} gave similar observations of peptide residues derived from α -casein when the tryptic digestion product of the same commercial sample of β -casein with 90% purity was used as the sample. The red shifts arising in the LSPR spectra

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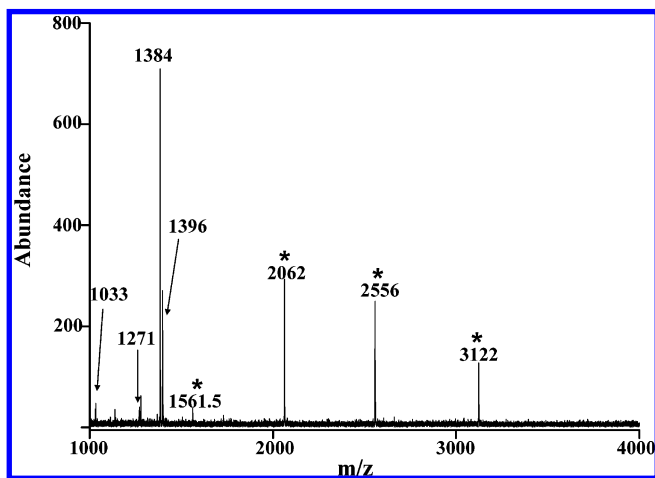


Figure 6. MALDI mass spectrum of the bare gold immobilized sensing substrate used for obtaining absorption band b in Figure 5b. 2,5-DHB was added onto the substrate before MALDI-MS analysis.

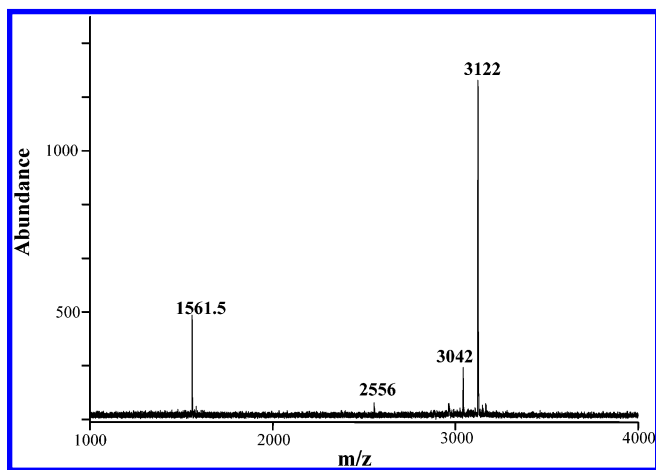


Figure 8. MALDI mass spectrum of the sensing substrate used to obtain the absorption band d in Figure 7. 2,5-DHB (30 mg/mL, 0.4 μ L) was applied on the substrate before MALDI-MS analysis.

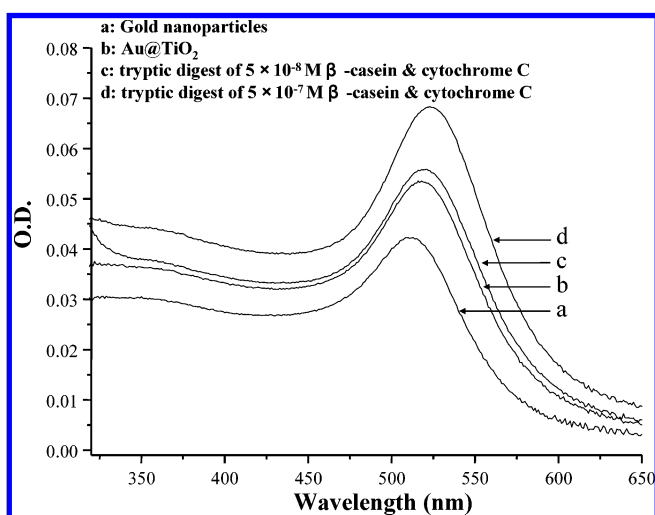


Figure 7. LSPR spectra of the glass slides. Slides (a) coated with gold nanoparticles (\sim 26 nm); (b) coated with Au@TiO₂ nanoparticles; (c) as (b) exposed to tryptic digest of equal concentrations of cytochrome *c* and β -casein at 5×10^{-8} M; (d) as (c) with concentration 5×10^{-7} M.

when using the bare gold nanoparticles immobilized glass slide as the LSPR sensing substrate were contributed from both of non-phosphopeptides and phosphopeptides. The results indicated that the sensing substrate coated with only bare gold nanoparticles has no specificity for phosphopeptides.

We further investigated the selectivity of this sensing method for a mixture of tryptic digest products of cytochrome *c* and β -casein. In Figure 7, the LSPR absorption spectrum, (a) is of the slide coated only with gold nanoparticles, while (b) is of the slide coated with Au@TiO₂ nanoparticles. Bands c and d correspond to the samples obtained using the LSPR sensing substrate to selectively trap the target species from the tryptic digest product of the protein mixture (cytochrome *c* and β -casein) at 5×10^{-8} and 5×10^{-7} M, respectively. The red shift is \sim 2 nm at 5×10^{-8} M increasing to 6 nm at 5×10^{-7} M. MALDI-MS was employed again to validate that the red shifts are due to the binding of phosphopeptides on the sensing substrate. Figure 8 displays the MALDI mass spectrum of the sensing substrate, which gave

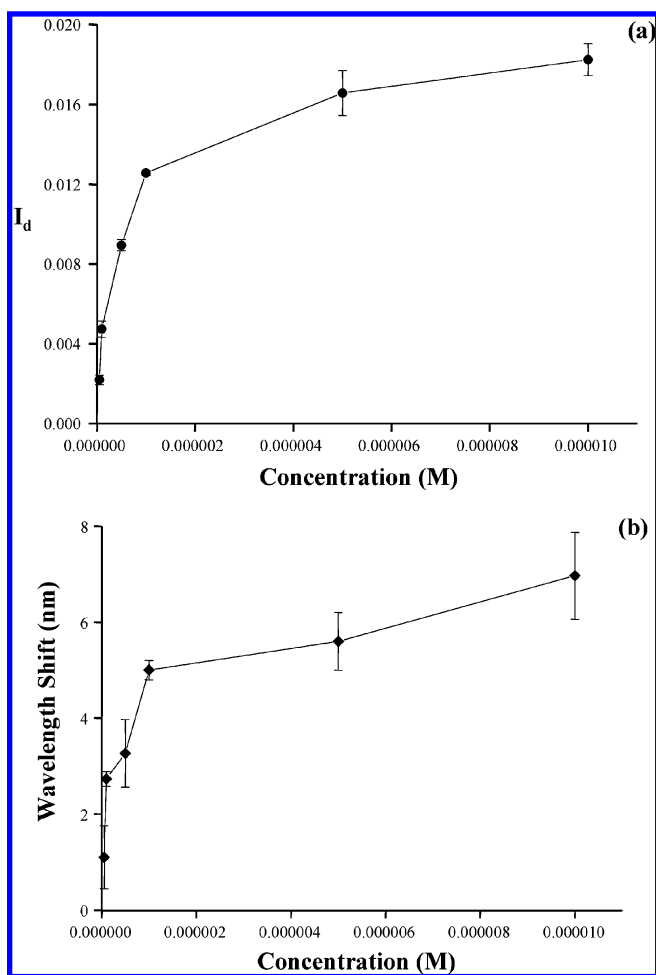


Figure 9. (a) Plot of the LSPR response (at 517 nm) of the tryptic digest product of β -casein as a function of its concentration. (b) Plot of the LSPR wavelength shift of the tryptic digest product of β -casein as a function of its concentration.

band d in Figure 7. That is, the sample solution contains the tryptic digest product of the mixture of cytochrome *c* (5×10^{-7} M) and β -casein (5×10^{-7} M). The peak at m/z 3122 (16–40) derived from β -casein dominates the mass spectrum. The doubly charged ion at m/z 1561.5 still appears, while the peak at m/z 3042

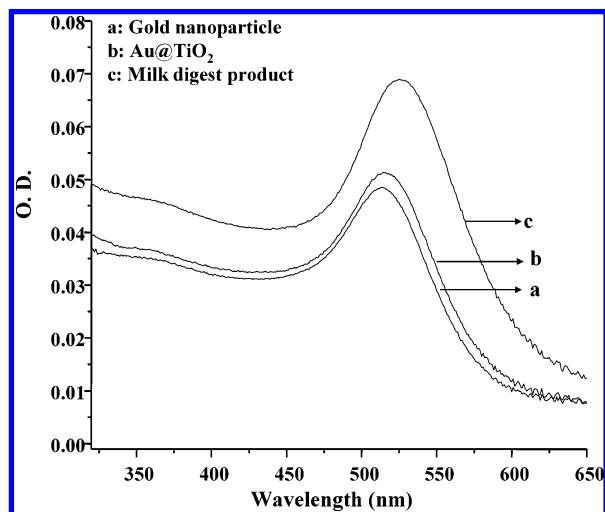


Figure 10. LSPR spectra of the glass slides. Slides (a) coated with gold nanoparticles (~ 26 nm); (b) coated with Au@TiO₂ nanoparticles; (c) as (b) with target species from the tryptic digest product of milk (1000-fold diluted).

corresponds to the loss of a phosphate group from the peak at m/z 3122. The peak at m/z 2556 (48–67) is also derived from β -casein. The results confirm that the red shifts in the LSPR spectrum are due to the binding of phosphopeptides on the LSPR sensing substrate. Furthermore, the results indicate that the sensing capacity of our approach toward phosphopeptides is quite good.

We also examined the relationship between the concentration and SPR responses. Figure 9a displays the curve by plotting the intensity difference (I_a) at 517 nm of the SPR absorption before and after depositing the sample, i.e. tryptic digest product of β -casein, on a sensing substrate as the function of the concentration of the tryptic digest product of β -casein. SPR responses increase as the increase of the concentration of the samples although there is no linear relationship between intensity differences and concentrations. The sensing chip seems saturated as the sample concentration is over 10^{-6} M. Figure 9b displays the curve by plotting the wavelength shift of the SPR absorption before and after depositing the sample, i.e., tryptic digest product of β -casein, on a sensing substrate as the function of the concentration of the tryptic digest product of β -casein. The pattern of the plot in Figure 9b is similar to what we observed in Figure 9a. There is only a slight red shift of the SPR absorption band as the concentration of the tryptic digestion product of β -casein is over 10^{-6} M.

Milk contains abundant proteins, including caseins. To investigate whether this approach can specifically trap phosphopeptides from such a complex sample, we subjected a tryptic digest product of milk (1000-fold diluted) to LSPR detection. In Figure 10, band a is the LSPR spectrum of the slide coated only with gold nanoparticles, while band b is of the slide with immobilized Au@TiO₂ nanoparticles, which show a ~ 3 -nm red shift after the spin-coating of titania on the gold nanoparticles. The absorption band c in Figure 10 is the SPR spectrum of the sample obtained using the LSPR sensing substrate to trap the target species from the tryptic digest product of milk. The absorption band c has ~ 7 -nm red shift from the absorption band b. MALDI-MS again confirmed that the

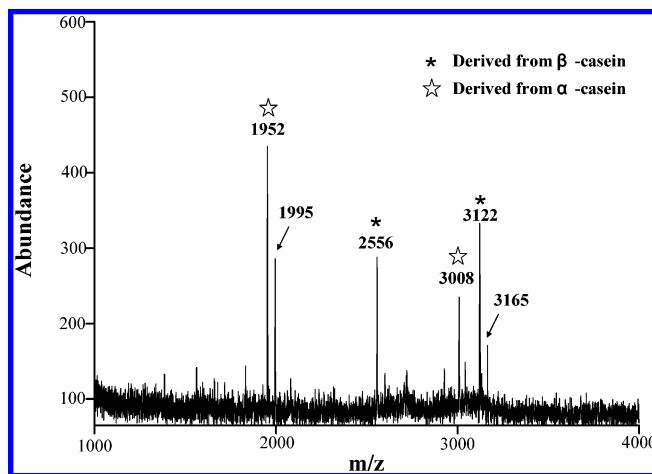


Figure 11. MALDI mass spectrum of the sensing substrate used to obtain absorption band c in Figure 10. 2,5-DHB (30 mg/mL, 0.4 μ L) was applied on the substrate before MALDI-MS analysis.

red shift is due to the binding of phosphopeptides. Figure 11 shows the MALDI mass spectrum of the sensing substrate used to obtain the absorption band c in Figure 10. The peaks at m/z 1952 (S1/104–119) and 3008 (S2/46–70) are derived from α -casein, while the peaks at m/z 2556 (48–67) and 3122 (16–40) are derived from β -casein. Although there are a few small peaks not from phosphoproteins, the results demonstrate that phosphopeptides can be detected in a very complex sample.

CONCLUSIONS

We have demonstrated a novel approach to the determination of phosphopeptides in complex samples using LSPR as the detection method. The combination of the LSPR technique with the affinity of titania in interacting with phosphopeptides makes this method quite promising. Au@TiO₂ nanoparticles immobilized on glass substrates have been shown capable of sensing phosphopeptides. To the best of our knowledge, the present approach is the first to combine the unique features of titania and gold nanoparticles in a LSPR biosensor. The high selectivity of titania for phosphopeptides makes this technique very efficient for rapid analysis without requiring tedious pretreatment. However, unidentified peaks with low intensities were observed in the MALDI mass spectra. This method provides a rapid screening method for phosphopeptides, but it requires alternative analysis method for further confirmation. This approach can be further improved if a sensing substrate with higher specificity for phosphopeptides is explored. This approach potentially can be applied for detection of phosphorylated species from very complex samples such as cell lysates.

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