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ARTICLE

Quantitative Detection of Glyphosate by Simultaneous Analysis of UV Spectroscopy and Fluorescence Using DNA-Labeled Gold Nanoparticles

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A sandwich-type immunosensor composed of antigen−double target/probe DNA-coated gold nanoparticles (NPs) was developed for the measurement of fluorescence intensity and quantitative analysis of single-stranded DNA based on the concentration of free glyphosate. The reaction between the antigen−double DNA−gold NPs and immobilized antibody on the substrate was carried out for 2 h. The results of the antigen−antibody reaction were measured on the basis of the fluorescence intensity obtained from comparison with the free antigens at concentrations of 0.01−100 μg mL^{-1} for the detection of immobilized antigen−double DNA−gold NPs. For the quantitative analysis based on the concentration of glyphosate(0.01−100 μg mL^{-1}), the immunosensor response also revealed the same detection range of glyphosate using DNA detection.

KEYWORDS: Immunosensor; gold nanoparticles; antigen−antibody; glyphosate; fluorescence; quantitative analysis

INTRODUCTION

Glyphosate is a postemergence nonselective herbicide that is used to control a wide variety of weeds (*1*). Due to its relatively low toxicity to mammals, glyphosate has become the most widely used herbicide. However, its widespread application has created problems regarding environmental contamination; therefore, its detection in crops, vegetables, and fruits has become increasingly important (*2*).

Rapid detection methods are required to control dangerous pollutants in various environments such as agricultural systems or groundwater. The analytical methods used for the detection of general pesticides usually employ equipment that measures the permissible level of pesticides; such methods include high-performance liquid chromatography (HPLC) (*3*), thin-layer chromatography (TLC) (*4*), and gas chromatography−mass spectroscopy (GC-MS) (*5*). Liquid chromatography (LC) and gas chromatography (GC) are widely used for this purpose due to their high sensitivity of detection and accuracy. However, these detection methods are very complex, time-consuming, and expensive and require bulky instrumentation (*6*). As a result, immunoassay-based detection techniques are being developed to detect toxic chemicals in the environment.

In recent years, immunoassays developed for the detection of toxic chemicals in environmental systems have been required to be highly specific, exhibit high sensitivity, and enable selective detection in a wide variety of fields, including drug delivery and biodefense (*7*, *8*). Immunoassays such as those used in medical diagnostics, food inspection, and biomedical research (*9*, *10*) have been applied to detection methods to enable rapid or low-cost detection, with the most useful techniques utilizing biomarkers to enable portability and simplicity (*11*−*20*). Despite the many advances in this field, the development of immunoassays is still in its early stage and can be applied to many more fields (*21*−*24*). A type of biolable amplification assay was applied to the biodetection of protein and DNA, and it was increased in sensitivity by polymerase chain reaction (PCR) (*25*−*28*). Other methods such as chemiluminescent (*29*), fluorescent (*30*), eletrochemical (*31*, *32*), inductively coupled plasma mass spectrometric (ICPMS) (*33*, *34*), atomic absorption spectrometric (*35*), electrochemiluminescent (*36*), micellar electrokinetic capillary chromatographic (*37*), and liquid phase binding (*38*) immunoassays have also been used to detect pollutants with high sensitivity. However, their detection times are usually long, and the procedures are expensive. This paper aimed to develop a novel immunosensor that could be used for the rapid and inexpensive detection of glyphosate as well as enhancement of sensitivity.

MATERIALS AND METHODS

Materials. Gold NPs (40 nm) were purchased from BB International (USA). Synthetic oligonucleotides with the following base sequences were purchased from Bionics (Korea): 5′-CGC ATT CAG GAT TGC ATG ATT GCC AAA AAA AAA A-NH_2-3′, amine-functionalized group DNA (target); 5′-CGC ATT CAG GAT TGC ATG ATT GCC AAA AAA AAA A-3′, nonfunctionalized group DNA (probe); 5′-GGC AAT CAT GCA ATC CTG AAT GCG AAA AAA AAA A-SH-3′, thiol-functionalized group DNA. Antigens of pesticide (glyphosate) and 3-aminopropyltriethoxysilane (3-APTES) were purchased from Sigma-Aldrich

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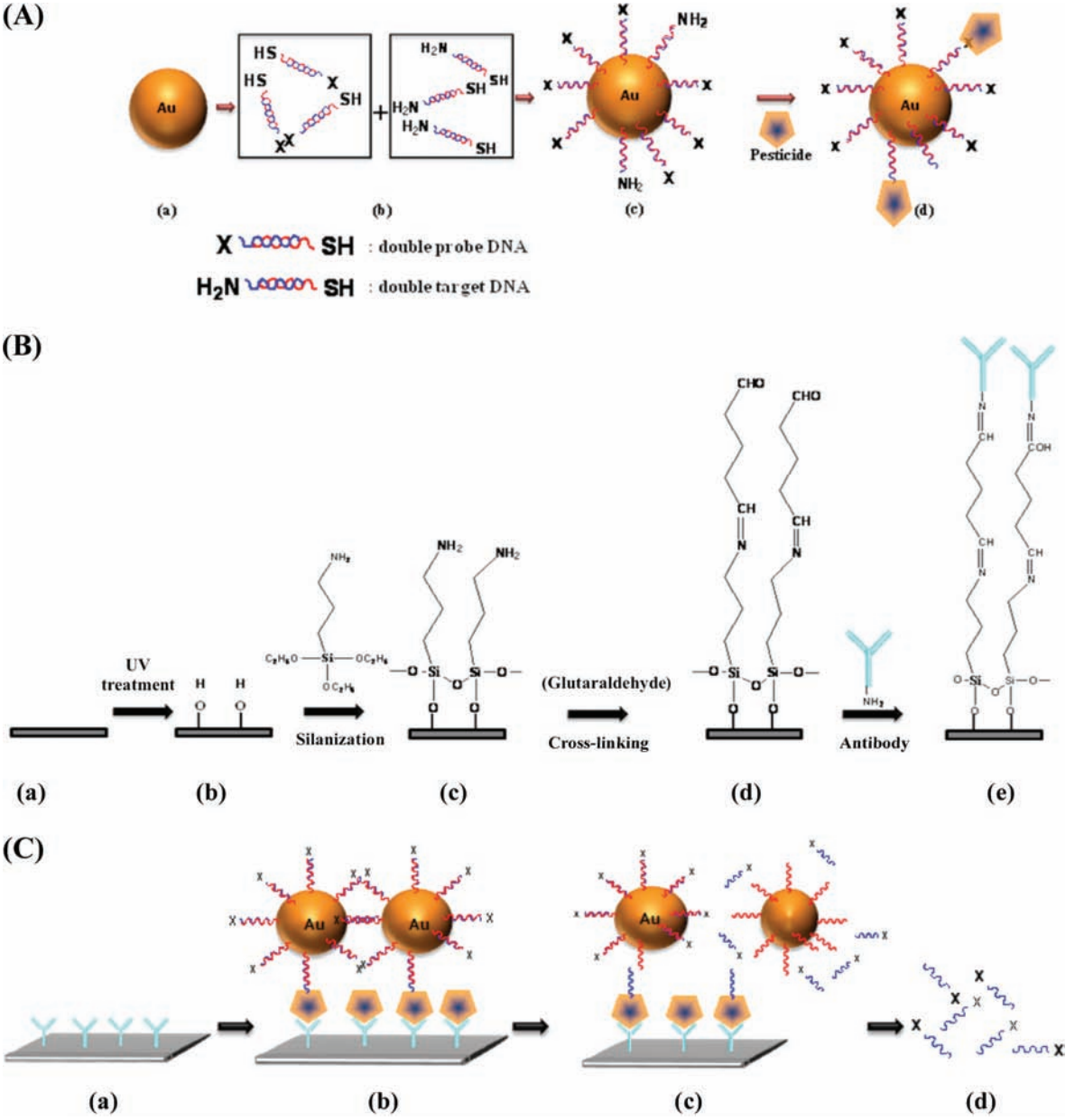

Figure 1. Novel immunoassay containing glyphosate−double DNA−gold NPs based on competitive inhibition reaction. (**A**) Antigen method (glyphosate immobilized onto double DNA−gold NP conjugate): (a) bare gold NPs; (b) double-probe/target DNAs; (c) double DNAs immobilized on gold NPs; (d) glyphosate immobilized on double DNA−gold NP conjugate. (**B**) Modification of surface of glass substrate: (a) glass substrate; (b) formation of −OH group on the glass substrate after UV treatment; (c) formation of $-NH_2$ group after silanization; (d) formation of −CHO group after reaction with glutaraldehyde; (e) immobilization of glyphosate antibody by amide bond. (**C**) Analysis of single-probe DNAs obtained by the capture of glyphosate−double DNA−gold NPs based on the competitive inhibition reaction: (a) glyphosate antibody on the glass substrate; (b) competitive inhibition reaction with the mixture solution (glyphosate−double DNA−gold NP and free glyphosate); (c) separation of double DNA−gold NPs; (d) single-probe DNA analysis at 260 nm.

(USA). Glyphosate polyclonal antibodies (rabbit host) were purchased from Abcam (USA) and Young In Frontier Co. (Korea). Glutaraldehyde was obtained from Fluka (USA).

Preparation of Sandwich-Type Antigen−Double DNA−Gold NPs. Double-target and probe DNAs were prepared using a single amine-functionalized group DNA (target), a single nonfunctionalized group DNA (probe), and a thiol-functionalized group DNA. Two microliters of 12.3 nM single amine-functionalized group DNA (target) solution and 2 μL of 12.3 nM single nonfunctionalized group DNA (probe) solution were mixed with 2 μL of 12.3 nM single thiol-functionalized group DNA by heating for 5 min at 95 °C in 96 μL of phosphate-buffered saline (PBS) at pH 7.4. Sixty microliters of double-probe DNA solution was then mixed with 30 μL of double-target DNA at a ratio of 2:1 for 1 min.

To modify gold NPs, 1 mL of gold NPs (0.01 wt %) was centrifuged for 20 min at 2000*g* and then washed in PBS buffer. The washed gold NPs were then added to the DNA mixture solution and shaken for 60 min at 4 °C to produce self-assembly monolayers.

For formation of antigen−double target/probe DNA−gold NPs, 90 μL of 1 mM glyphosate in PBS buffer (pH 7.4) for the antigen was added to induce DNA recombination with the gold NP solution. The mixture solutions were then shaken for 2 h at 4 °C for immobilization. Finally, the solutions were centrifuged at 2000*g*, after which they were thoroughly washed in PBS solution to eliminate unimmobilized glyphosate (**Figure 1A**).

Preparation of Captured Antibody Dots onto Modified Glass Substrate. Modified aldehyde groups on a glass substrate were prepared by following standard methods (*39*). A glass substrate was cleaned by soaking in 100 mL of H_2O_2 (35%) at 25 °C for 2 h, followed by washing with distilled water. A −OH group was introduced to the glass surface by treatment with UV light (at 184 nm, intensity = 15−18 mW cm^{-2}) for 10 min, followed by drying at 120 °C for 2 h. Substrates were then treated

with 10 mL of 3-APTES in 90 mL of acetone at 50 °C for 2 h to induce the amino groups to adhere to the substrate. The slide was then washed with distilled water and dried at 60 °C for 2 h. The substrates were modified with 400 mL of phosphate buffer (pH 7.0) by soaking the samples in 2.6 M glutaraldehyde at 4 °C for 1 h and then at 20 °C for 1 h (**Figure 1B**).

To immobilize glyphosate antibody, the modified substrates were washed with distilled water. Then, 0.3 μL of 1 mg mL^{-1} glyphosate antibodies in PBS buffer (pH 7.4) was immobilized on dots formed in the detection zone of the substrate by the hand spot method at room temperature for 2 h as detailed in ref *40*. Finally, the substrates were washed in PBS solution (pH 7.4).

Immunoassay for Detection of Fluorescence Intensity. For the detection of fluorescence intensity, immobilized antigen on the surface of the gold NPs was added to each of the 180 μL solutions containing free glyphosate at concentrations ranging from 100 to 0.01 μg mL^{-1}. Mixture solutions were prepared, wetted, and incubated at 25 °C for 2 h on the antibody dots. After the reaction, the substrates were washed two times in PBS buffer (pH 7.4) containing 0.3% (v/v) Tween 20. Washed substrates were assessed by measuring the fluorescence intensity of immobilized gold NPs through competitive immunoreactions.

Measurement for Detection of Single-Probe DNA. Different amounts of gold NPs on the functional substrates were heated at 95 °C for 5 min in 1 mL of PBS (pH 7.4) after removal of the glass substrate. The solutions containing single-probe DNAs were measured using a UV spectrophotometer (UV Mini-1240, Shimadzu, Japan) at 260 nm (**Figure 1C**). Concentrations of the solutions were then calculated from a plot constructed using a calibration curve.

RESULTS AND DISCUSSION

Gold NP-Based Immunoassay of Glyphosate. Glyphosate was subjected to immunoassay based on competitive immunoreactions in which developed glyphosate−dsDNA−gold NP conjugates compete with free glyphosate in the sample for available antibody binding sites on the substrate. Conjugated antigens of glyphosate on the surface of gold NPs were immobilized on the antibody dots by an antigen−antibody interaction, as amine-functionalized groups were present in the target DNA and attached to the aldehyde groups of glyphosate by imine reactions. To investigate the detection of stable glyphosate−dsDNA−gold NP conjugates, the fluorescence of glyphosate−dsDNA−gold NP conjugates was compared to that of free glyphosate at concentratoins ranging from 100 to 0.01 μg mL^{-1} (**Figure 2**).

Figure 2A shows the calibration curve of the fluorescence of glyphosate−dsDNA−gold NPs under optimum conditions. The linear dependence of the concentration of free glyphosate yielded a regression equation of intensity with a correlation coefficient of 0.95. When the concentrations of free glyphosate decreased to 0.01 μg mL^{-1}, the amount of immobilized free glyphosates on the surface antibody dots likewise decreased. In the results, the amount of immobilized antigen−double DNA−gold NPs on the surfaces of the antibody dots was increased at the fluorescence intensity of the first detection. Brighter fluorescent images were also observed (**Figure 2B**). Therefore, gold NPs were immobilized through competitive immunoreactions involving free glyphosates. Specifically, the assay was composed of antibodies containing amine functional groups that immobilized the aldehyde functional group of the substrate (imine reaction). Both free glyphosates and gold NPs competitively reacted with the immobilized antibodies.

In addition, single-probe DNA was separated from DNA-conjugated gold NPs on the surfaces of the antibody dots by heating the substrate in PBS buffer (pH 7.4) solution. After removal from the substrate, the concentrations of single-probe DNAs with free glyphosate were measured using a UV spectrophotometer. The optical density of the single-probe DNA that was separated from double-probe DNA was measured to identify the amount of free glyphosate bound to the gold particles. Therefore, an indirect

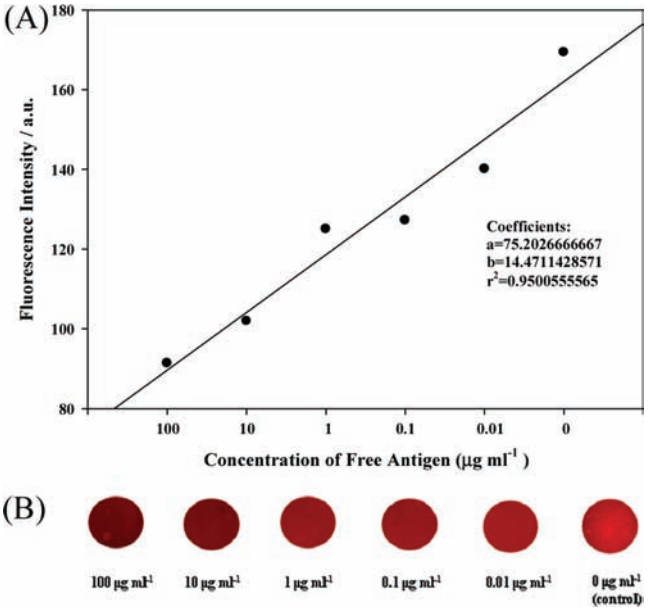

Figure 2. Fluorescence intensity analysis: (**A**) calibration plot for the immunoassay on the glass substrate; (**B**) fluorescence images of immobilized glyphosate−double DNA−gold NPs mixed with free glyphosate from 0.01 to 100 μg mL^{-1} on the glass substrate. The images shown were immobilized by antigen−antibody reaction (1 s of exposure time and 200 of sensitivity).

detection method was used to measure the amount of single-probe DNA instead of free glyphosates, which led to increased sensitivity.

Two different types of double-target/probe DNAs were utilized to immobilize and separate glyphosate antigen, followed by quantitative analysis. One type was functionalized using single-thiolated DNA and nonfunctionalized DNA (probe) having a complementary sequence, whereas the other employed thiolated DNA and amine-functionalized DNA (target). The process can be reversed when the temperature is raised, which results in the breaking of hydrogen bonds, that is, melting of the DNA double helix. The complementary sequence of the DNA was designed so that single-target and probe DNA were specifically bound at 65−67 °C, because the annealing temperature enhanced selectivity. In addition, the repeated sequence of poly A was used as a linker between the gold NPs and glyphosate antigen for the reaction of antigen−double DNA−gold NPs. The binding activity of the mismatched sequences of the single-target DNA and probe DNA was compared with that of a complementary sequence containing thiol-functionalized group DNA. Two kinds of double DNAs (target, probe) were induced to undergo DNA recombination. The mixed solution with double-target DNA and double-probe DNA was conjugated on the surface of the gold NPs at a thiol-functionalized group. The antigen of glyphosate was then immobilized using double-target DNAs. The term "DNA recombination" indicates that DNA moved from a thiol-functionalized DNA molecule to an amine-functionalized (target) or nonfunctionalized (probe) DNA molecule. The thiol-functionalized DNA molecule was complementary to the amine-functionalized (target) and nonfunctionalized (probe) DNA molecules, which mediated the antigen−antibody interaction for the first detection and quantitative analysis for the second detection. The double DNAs were made from two kinds of DNA (target DNA and probe DNA) by recombination.

The use of gold NPs also contributed to the high sensitivity of the immunoassay. The size of the gold NPs was approximately 40 nm (*41*). Some advantages of using gold NPs are convenient

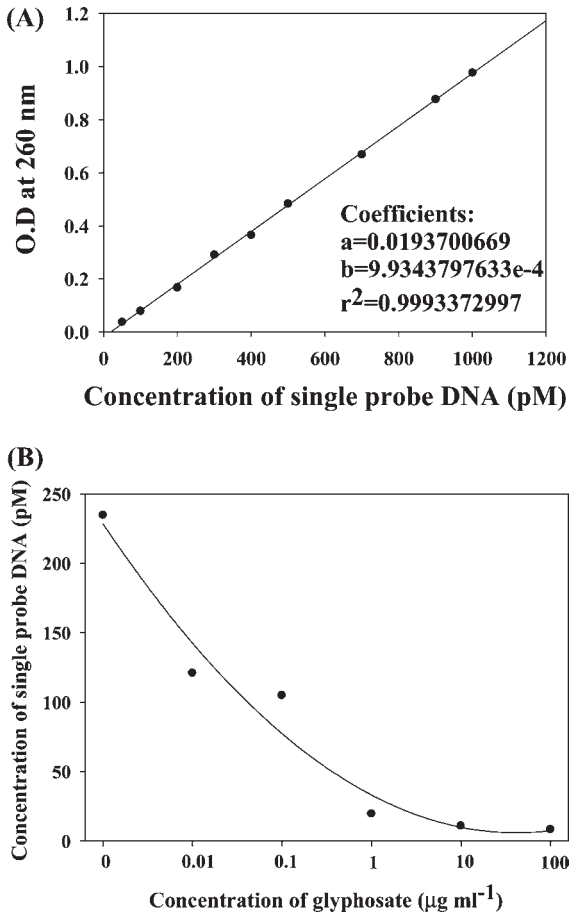

Figure 3. Single-probe DNA analysis: (**A**) calibration curve describing the optical density at various concentrations of single-probe DNA by UV spectrometry at 260 nm; (**B**) glyphosate analyzed at concentrations ranging from 0.01 to 100 μg mL^{-1} by the novel immunoassay.

preparation of desired sized NPs, high monodispersity in aqueous phase, and ease of producing conjugates with biomolecules that can function as detector reagents (*42*). Previous works have shown that the high sensitivity offered by gold NPs is due to dispersion, density, and a higher surface-to-volume ratio than other metal particles (*43*, *44*). Besides, gold NP-modified electrode surfaces can be prepared by covalently binding gold NPs with solid surfaces modified with surface functional groups (−SH) of self-assembled monolayers (SAMs) (*45*−*48*). Therefore, gold NPs were applied to the immunoassay to enable the detection of free glyphosate at concentrations of 0.01 μg mL^{-1}.

Sensitivity Enhancement of Glyphosate. Hand spotting was utilized for the substrate. Uniformly sized antibody dots are essential for fluorescence intensity and quantitative analysis. However, the results of this study were confirmed on the basis of the concentrations of free antigen and single probe DNA, although nonidentical results could have resulted from mistakes during spotting. A constant concentration of antibody dot affected the analysis of fluorescence, whereas the size of antibodies affected the quantitative analysis. The intensity of fluorescence in the first detection decreased when free antigen and gold particles were immobilized in the presence of nonuniform concentrations of antibody. Therefore, a PBS (pH 7.4) buffer solution was utilized to make the concentrations of incubated antibody dots uniform. Otherwise, the accuracy of the quantitative analysis for the second detection decreased when the size of the dots was nonuniform. After immobilization of antigen−double DNA−gold NPs on the substrate, the single-probe DNAs were separated from double-probe DNAs on the surfaces of the gold NPs by heating the substrate in PBS buffer solution. The amount of single-probe DNAs was then measured through quantification of free glyphosate using a UV spectrophotometer at 260 nm. The mechanism of the immunoassay using competitive immunoreactions was mediated through the detection of free glyphosate during the time at which the concentration of single-probe DNA increased. It seems reasonable to conclude that the immunoassay was able to detect pesticides at a concentration of 0.01 μg mL^{-1} (**Figure 3**).

The detection limits of the previously developed analytical methods were similar to those of the immunoassay that was developed in this study; however, they required a longer time to detect the pesticides. In addition, the novel immunoassay developed here can be applied to the detection of other pesticides and target materials containing carboxyl groups.

In conclusion, a novel immunoassay containing pesticides−dsDNA−gold NPs was developed on the basis of competitive immunoreaction of antibodies. The method comprised capturing glyphosate−dsDNA−gold NPs and then detecting single-probe DNA to measure the level of free glyphosates (0.01−100 μg mL^{-1}). This immunoassay enabled detection of several pesticides at concentrations as low as 0.01 μg mL^{-1} within 2 h. The immunoassay described here can provide rapid sample analysis with decreased laboratory costs and is applicable to other pesticides containing carboxyl groups. Therefore, it is suggested that this immunoassay be used as an immunosensor for the accurate detection of free antigen concentrations in the range of 0.01 μg mL^{-1}. The primary advantage of this method is that it allows detection of samples using high concentrations of single-probe DNAs. In the same way, further experiments for the immunosensor characterization of interest such as the food safety and disease diagnosis fields will make the best use of the assay.

LITERATURE CITED

(1) Coutinho, C. F. B.; Coutinho, L. F. M.; Mazo, L. H.; Nixdorf, S. L.; Camara, C. A. P.; Lanças, F. M. Direct determination of glyphosate using hydrophilic interaction chromatography with coulometric detection at copper microelectrode. *Anal. Chim. Acta* **2007**, *592*, 30−35.

(2) Khrolenko, M. V.; Wieczorek, P. P. Determination of glyphosate and its metabolite aminomethylphosphonic acid in fruit juices using supported-liquid membrane preconcentration method with high-performance liquid chromatography and UV detection after derivatization with *p*-toluenesulphonyl chloride. *J. Chromatogr., A* **2005**, *1093*, 111−117.

(3) Moltó, J. C.; Picó, Y.; Font, G.; Mañes, J. Determination of triazines and organophosphorus pesticides in water samples using solid-phase extraction. *J. Chromatogr., A* **1991**, *555*, 137−145.

(4) Sacchero, G.; Sarzanini, C.; Mentasti, E. On-line preconcentration and ion chromatography of triazine compounds. *J. Chromatogr., A* **1994**, *671*, 151−157.

(5) Hernández, F.; Hidalgo, C.; Sancho, J. V.; López, F. J. Coupled-column liquid chromatography applied to the trace-level determination of triazine herbicides and some of their metabolites in water samples. *Anal. Chem.* **1998**, *70*, 3322−3328.

(6) Panshin, S. Y.; Carter, D. S.; Bayless, E. R. Analysis of atrazine and four degradation products in the pore water of the vadose zone, central Indiana. *Environ. Sci. Technol.* **2000**, *34*, 2131−2137.

(7) Ferrari, M. Cancer nanotechnology: opportunities and challenges. *Nat. Rev. Cancer* **2005**, *5*, 161−171.

(8) Stoeva, S. I.; Lee, J.-S.; Smith, J. E.; Rosen, S. T.; Mirkin, C. A. Multiplexed detection of protein cancer markers with biobarcoded nanoparticle probes. *J. Am. Chem. Soc.* **2006**, *128*, 8378−8379.

(9) Cho, E. C.; Jang, B. O.; Kim, E. J.; Koo, K. -K. Characterization of a carbon composite electrode for an electrochemical immunosensor. *Korean J. Chem. Eng.* **2008**, *25*, 548−552.

(10) Wong, Y. Y.; Ng, S. P.; Ng, M. H.; Si, S. H.; Yao, S. Z.; Fung, Y. S. Immunosensor for the differentiation and detection of *Salmonella*

species based on a quartz crystal microbalance. *Biosens. Bioelectron.* **2002**, *17*, 676−684.
(11) Cao, Y. W. C.; Jin, R.; Mirkin, C. A. Nanoparticles with raman spectroscopic fingerprints for DNA and RNA detection. *Science* **2002**, *297*, 1536−1540.
(12) Cui, Y.; Wei, Q.; Park, H.; Lieber, C. M. Nanowire nanosensors for highly sensitive and selective detection of biological and chemical species. *Science* **2001**, *293*, 1289−1292.
(13) Gómez-Hens, A.; Fernández-Romero, J. M.; Aguilar-Caballos, M. P. Nanostructures as analytical tools in bioassays. *Trends Anal. Chem.* **2008**, *27*, 394−406.
(14) Han, M.; Gao, X.; Su, J. Z.; Nie, S. Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. *Nat. Biotechnol.* **2001**, *19*, 631−635.
(15) Li, X.-M.; Yang, X.-Y.; Zhang, S.-S. Electrochemical enzyme immunoassay using model labels. *Trends Anal. Chem.* **2008**, *27*, 543−553.
(16) Niemeyer, C. M.; Ceyhan, B. DNA-directed functionalization of colloidal gold with proteins. *Angew. Chem., Int. Ed.* **2001**, *40*, 3685−3688.
(17) Rosi, N. L.; Mirkin, C. A. Nanostructures in biodiagnostics. *Chem. Rev.* **2005**, *105*, 1547−1562.
(18) Schäferling, M.; Wolfbeis, O. S. Europium tetracycline as a luminescent probe for nucleoside phosphates and its application to the determination of kinase activity. *Chem.—Eur. J.* **2007**, *13*, 4342−4349.
(19) Taton, T. A.; Mirkin, C. A.; Letsinger, R. L. Scanometric DNA array detection with nanoparticle probes. *Science* **2000**, *289*, 1757−1760.
(20) Wang, J.; Liu, G.; Munge, B.; Lin, L.; Zhu, Q. DNA-based amplified bioelectronic detection and coding of proteins. *Angew. Chem., Int. Ed.* **2004**, *43*, 2158−2161.
(21) Cho, I.-H.; Paek, E.-H.; Lee, H.; Choi, J.-W.; Paek, S.-H. Site-directed immobilization of antibody onto solid surfaces for the construction of immunochip. *Biotechnol. Bioprocess Eng.* **2004**, *9*, 112−117.
(22) Lin, Z. H.; Wu, M.; Wolfbeis, O. S.; Schäferling, M. A novel method for time-resolved fluorimetric determination and imaging of the activity of peroxidase, and its application to an enzyme-linked immunosorbent assay. *Chem.—Eur. J.* **2006**, *12*, 2730−2738.
(23) Munge, B.; Liu, G.; Collins, G.; Wang, J. Multiple enzyme layers on carbon nanotubes for electrochemical detection down to 80 DNA copies. *Anal. Chem.* **2005**, *77*, 4662−4666.
(24) Wang, J.; Ibáñez, A.; Chatrathi, M. P. On-chip integration of enzyme and immunoassays:simultaneous measurements of insulin and glucose. *Am. Chem. Soc.* **2003**, *125*, 8444−8445.
(25) Hill, H. D.; Mirkin, C. A. The bio-barcode assay for the detection of protein and nucleic acid targets using DTT-induced ligand exchange. *Nat. Protoc.* **2006**, *1*, 324−336.
(26) Nam, J.-M.; Stoeva, S. I.; Mirkin, C. A. Bio-bar-code-based DNA detection with PCR-like sensitivity. *J. Am. Chem. Soc.* **2004**, *126*, 5932−5933.
(27) Oh, B.-K.; Nam, J.-M.; Lee, S. W.; Mirkin, C. A. A fluorophore-based bio-barcode amplification assay for proteins. *Small* **2006**, *2*, 103−108.
(28) Stoeva, S. I.; Lee, J.-S.; Thaxton, C. S.; Mirkin, C. A. Multiplexed DNA detection with biobarcoded nanoparticle probes. *Angew. Chem., Int. Ed.* **2006**, *45*, 3303−3306.
(29) Fu, Z.; Yan, F.; Liu, H.; Yang, Z.; Ju, H. Channel-resolved multi-analyte immunosensing system for flow-through chemiluminescent detection of α-fetoprotein and carcinoembryonic antigen. *Biosens. Bioelectron.* **2008**, *23*, 1063−1069.
(30) Matsuya, T.; Tashiro, S.; Hoshino, N.; Shibata, N.; Nagasaki, Y.; Kataoka, K. A core-shell-type fluorescent nanosphere possessing reactive poly(ethylene glycol) tethered chains on the surface for zeptomole detection of protein in time-resolved fluorometric immunoassay. *Anal. Chem.* **2003**, *75*, 6124−6132.
(31) Qiang, Z.; Yuan, R.; Chai, Y.; Wang, N.; Zhuo, Y.; Zhang, Y.; Li, X. A new potentiometric immunosensor for determination of α-fetoprotein based on improved gelatin−silver complex film. *Electrochim. Acta* **2006**, *51*, 3763−3768.
(32) Zhang, S. S.; Zhong, H.; Ding, C. F. Ultrasensitive flow injection chemiluminescence detection of DNA hybridization using signal DNA probe modified with Au and CuS nanoparticles. *Anal. Chem.* **2008**, *80*, 7206−7212.
(33) Hu, S. H.; Zhang, S. C.; Hu, Z. C.; Xing, Z.; Zhang, X. R. Detection of multiple proteins on one spot by laser ablation inductively coupled plasma mass spectrometry and application to immuno- microarray with element-tagged antibodies. *Anal. Chem.* **2007**, *79*, 923−929.
(34) Zhang, S. C.; Zhang, C.; Xing, Z.; Zhang, X. R. Simultaneous determination of α-fetoprotein and free β-human chorionic gonadotropin by element-tagged immunoassay with detection by inductively coupled plasma mass spectrometry. *Clin. Chem.* **2004**, *50*, 1214−1221.
(35) Wang, G. L.; Yuan, J. L.; Gong, B. L.; Matsumoto, K.; Hu, Z. D. Immunoassay by graphite furnace atomic absorption spectrometry using a metal chelate as a label. *Anal. Chim. Acta* **2001**, *448*, 165−172.
(36) Blackburn, G. F.; Shah, H. P.; Kenten, J. H.; Leland, J.; Kamin, R. A.; Link, J.; Peterman, J.; Powell, M. J.; Shah, A.; Talley, D. B. Electrochemiluminescence detection for development of immunoassays and DNA probe assays for clinical diagnostics. *Clin. Chem.* **1991**, *37*, 1534−1539.
(37) Wang, R. Y.; Lu, X. N.; Ma, W. Y. Non-competitive immunoassay for α-fetoprotein using micellar electrokinetic capillary chromatography and laser-induced fluorescence detection. *J. Chromatogr., B* **2002**, *779*, 157−162.
(38) Kawabata, T.; Watanabe, M.; Nakamura, K.; Satomura, S. Liquid-phase binding assay of α-fetoprotein using DNA-coupled antibody and capillary chip electrophoresis. *Anal. Chem.* **2005**, *77*, 5579−5582.
(39) Lee, D. H.; Kim, J. M.; Shin, H. Y.; Kang, S. W.; Kim, S. W. Biodiesel production using a mixture of immobilized *Rhizopus oryzae* and *Candida rugosa* lipases. *Biotechnol. Bioprocess. Eng.* **2006**, *11*, 522−525.
(40) Ferguson, C. G.; James, R. D.; Bigman, C. S.; Shepard, D. A.; Abdiche, Y.; Katsamba, P. S.; Myszka, D. G.; Prestwich, G. D. Phosphoinositide-containing polymerized liposomes: stable membrane-mimetic vesicles for protein−lipid binding analysis. *Bioconjugate Chem.* **2005**, *16*, 1475−1483.
(41) Ding, C.; Zhang, Q.; Zhang, S. An electrochemical immunoassay for protein based on bio bar code method. *Biosens. Bioelectron.* **2009**, *24*, 2434−2440.
(42) Lyubavina, I. A.; Zinchenko, A. A.; Salomatina, I. S.; Zherdev, A. V.; Dzantiev, B. B. An immunochromatographic assay of 2,4-dichlorophenoxyacetic acid and simazine using monoclonal antibodies labeled with colloidal gold. *Russian J. Bioorg. Chem.* **2004**, *30*, 178−183.
(43) He, L.; Musick, M. D.; Nicewarner, S. R.; Salinas, F. G.; Benkovic, S. J.; Natan, M. J.; Keating, C. D. Colloidal Au-enhanced surface plasmon resonance for ultrasensitive detection of DNA hybridization. *J. Am. Chem. Soc.* **2000**, *122*, 9071−9077.
(44) Liu, T.; Tang, J.; Jiang, L. The enhancement effect of gold nanoparticles as a surface modifier on DNA sensor sensitivity. *Biochem. Biophys. Res. Commun.* **2004**, *313*, 3−7.
(45) Blonder, R.; Sheeney, L.; Willner, I. Three-dimensional redox-active layered composites of Au−Au, Ag−Ag and Au−Ag colloids. *Chem. Commun.* **1998**, *13*, 1393−1394.
(46) Fishelson, N.; Shkrob, I.; Lev, O.; Gun, J.; Modestov, A. D. Studies on charge transport in self-assembled gold-dithiol films: conductivity, photoconductivity, and photoelectrochemical measurements. *Langmuir* **2001**, *17*, 403−412.
(47) Grabar, K. C.; Freeman, R. G.; Hommer, M. B.; Natan, M. J. Preparation and characterization of Au colloid monolayers. *Anal. Chem.* **1995**, *67*, 735−743.
(48) Grabar, K. C.; Smith, P. C.; Musick, M. D.; Davis, J. A.; Walter, D. G.; Jackson, M. A.; Guthrie, A. P.; Natan, M. J. Kinetic control of interparticle spacing in Au colloid-based surfaces:rational nanometer-scale architecture. *J. Am. Chem. Soc.* **1996**, *118*, 1148−1153.

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