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SPR-based Antibody-Antigen Interaction for Real Time Analysis of Carbamate Pesticide Residues

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Abstract This research was conducted to develop a quick and sensitive method of detecting carbamate residues using the immobilization of antibody-antigen interactions with surface plasmon resonance (SPR). We have used commercialized surface plasmon resonance equipment (Biacore 3000). The antibody used for the immunoassay was specific for glutathione-S-transferase (GST) and the antigens included several carbamate pesticides (carbofuran, carbaryl, and benfuracarb). When antigens were applied to the protein GST, the detection limit was 2 ng/mL of carbamate pesticide. The fabricated protein GST maintained its activity for over 200 measurements. Thus we determined that the SPR biosensors could detect the specific reversible binding of a reactant in solution to a binding partner immobilized on the surface of the sensor and allow real-time detection and monitoring.

Keywords: biosensor, surface plasmon resonance (SPR), carbamate, pesticide residue

Introduction

Pesticides are raising public concerns around the world, because they cause environmental pollution and damage human health (1,2). Each country restricts pesticide residues by applying safety and usage standards so that they can control the amount of pesticide used in order to secure the safety of agricultural products.

The typical methods of analyzing residual pesticides are GC, HPLC, and GC/MS, in which pesticides are extracted and purified in a suitable organic solvent from a titrated, fixed amount of samples. In general, these methods are highly sensitive and accurate. On the other hand, they are costly, time and labor consuming, and require expensive equipment and skillful management (3-5).

Techniques and equipment suitable for the rapid inspection of agricultural products are preferable. Therefore, biosensors for the rapid detection of residual pesticides should be small, light, and cost-effective so that anyone can operate the equipment and detect residual pesticides easily.

In the past several years there has been increasing interest in detecting residual pesticides in an efficient way. To achieve this goal, antibodies directed at relevant environmental pollutants have been used for sensitive pesticide detection. Immunochemical systems are considered to be a cost-effective complement to chromatographic methods. Most approaches have used pesticidal action, the result of the inhibition of acetylcholinesterase (AChE) at nerve endings (cholinergic synapses) (6-8). Disk type detectors on which AChE protein is adsorbed have been developed and used (9,10). A spectroscopic method was also used to detect an ethylene bisdithyo carbamate (EBDC) germicide (11). In addition, an optical biosensor was constructed for the detection of captan and organophosphorus compounds, the major components in pesticides, by the inhibition of AChE and glutathione-S-transferase (GST) (12).

Recently, surface plasmon resonance (SPR)-based biosensors have been applied to the analysis of organophosphate pesticides in agriculture. The assessment of low intra- and inter-day coefficients of variation and a great number of regeneration cycles have proven the precision of SPR measurements and the reusability of the sensor surface, indicating the robustness of the method. The biosensing surface could be reused during 190 assay cycles without any significant loss of specific binding (13).

The objectives of this study were to find an immunological technology that could detect carbamate pesticides in buffer solution with very low detection limits using surface plasmon resonance, and to evaluate the measurement sensitivity and whether it is applicable to developing miniaturized biosensors. We also describe atomic force microscopy (AFM) images for analyzing the conditions of antibody immobilization on the surface of the gold chip.

Materials and Methods

Chemicals and antibodies Commercial glutathione S-transferase (GST) (EC 2.5.1.18, 1 mg plot/mL, from *Schistosoma japonicum*; Sigma-Aldrich Co., St Louis, MO, USA) and standard carbamate pesticides (carbofuran, carbaryl, and benfuracarb; Sigma-Aldrich Co.) were used for antibody-antigen reactions. Working standard solutions were prepared daily by dilution in 10 mM phosphate-buffered saline (PBS) solution.

A commercial carboxylated dextran matrix based (CM5) sensor chip with dextran spread on the surface (Biacore AB, Uppsala, Sweden), and an amine coupling kit with 70% of glycerol, BIAdesorb solution 1, BIAdesorb solution 2 and 1 M ethanolamine pH 8.5 (Biacore AB) were used for the immobilization of antibody. Common chemicals used in sensor surface immobilization were 100 mM N-

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hydroxysuccinimide (NHS) (Biacore AB), and 400 mM N'-ethyl-N-(3-dimethylamino-propyl) carbodimide hydrochloride (EDC) (Biacore AB).

SPR equipment SPR is an extension of evanescent wave sensing occurring when plane-polarized light hits a metal film with a critical incidence angle (θ) under total internal reflection conditions. Surface plasmons are electromagnetic waves that propagate along the interface between a metal and a dielectric material such as organic films (14) (Fig. 1).

Carbamate pesticide measurements in clean water were performed with SPR equipment (Biacore 3000; Biacore AB). The polarized light emitted by a 760 nm laser beam illuminates the gold-coated sensing surface, and reflected beams are detected by a spectrometer through a collecting fiber. Changes in the intensity of the reflected light indicate changes in mass on the surface. The SPR signal is then amplified and finally converted to a digital format. Real time changes in refractive index due to binding events occurring at the sensor surface can be monitored continuously (Fig. 2).

Immunoassay Immunoassay is usually used for detecting and quantitating substances such as peptides, proteins, antibodies, and hormones. An antibody must be immobilized to a solid surface. The antibody is complexed with an antigen that is linked to an enzyme. Detection is accomplished by incubating this enzyme-complex with a substrate that produces a detectable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction. We initially performed a pre-concentration test to find the optimum pH value of the buffer. Immobilization was then performed using the amine-coupling method. Surface coverage was examined by AFM image analysis. And finally pesticide concentrations were detected by immunoassay.

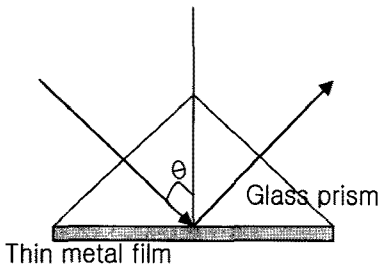

Fig. 1. Geometry used to excite surface plasmons.

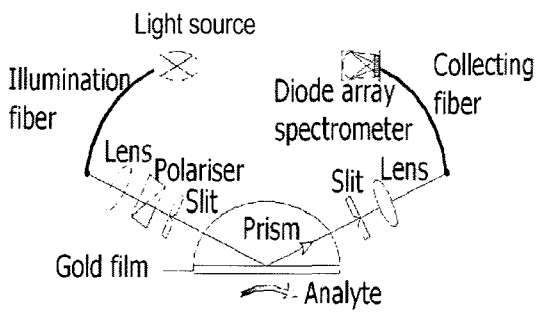

Fig. 2. Schematic of the SPR device.

Preconcentration testing Preconcentration tests were carried out to select the optimum pH buffer so that GST protein could be immobilized on the sensor surface easily. Ten mM sodium acetate buffer samples were prepared ranging from pH 7.0 to 3.5. GST was dissolved in each pH buffer at 50 μg/mL, and tested. Ten mM NaOH was then applied to the sensor surface to immobilize antibody for 1 min.

Enzyme immobilization There are many methods to immobilize ligand at the sensor surface. The amine coupling method was used to assure the regeneration and reusability of the sensor surface and achieve covalent linkage between sodium acetate and the sensor surface under non-denaturing conditions. First, a mixture of NHS/EDC (0.1/0.4 M in water) was pumped over the gold-coated sensor surface for a period of 7 min. EDC converts the carboxylic acid of the alkanethiol into reactive intermediates (NHS esters) which react with the free amine groups of the GST (50 μg/mL in 10 mM sodium acetate buffer, pH 4.5 selected by preconcentration test) protein conjugate. The immobilization process concluded with the blocking of the modified surface by using 1 M ethanolamine, pH 8.5. This procedure ensures both the elimination of the conjugate non-covalently bound to the layer and the deactivation of all unreacted NHS-esters remaining on the sensor surface. Running and dilution buffers (10 mM HEPES-NaOH, pH 7.4) were used in all binding experiments and for regeneration (Fig. 3).

AFM image analysis and immunological testing AFM images provide information of surface topological changes to identify captured proteins on protein arrays (15). Label-free methods such as SPR are capable of detecting and quantifying bound proteins onto arrays by using changes in the refractive index of the surface (16,17). Therefore, we took photographs of the antibody immobilization and analyzed them.

Carbamate pesticides (carbofuran, carbaryl, and benfuracarb) were prepared for immunological testing by dilution from 20 to 0.002 μg/mL (Table 1). There are 2 principal assay formats for concentration analysis using SPR: direct

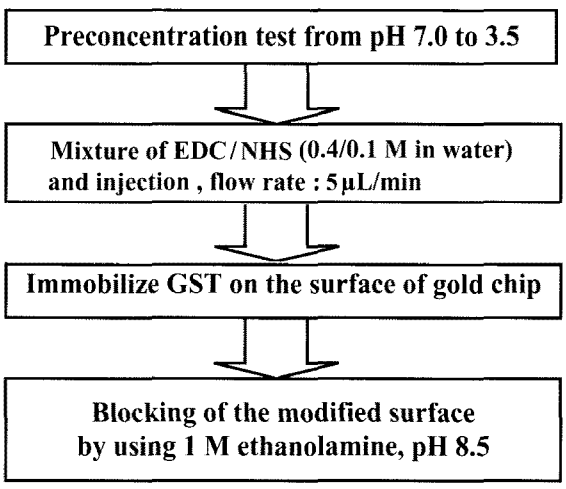

Fig. 3. Protocol for antibody immobilization on the gold chip surface.

Table 1. Concentration levels of each sample

Samples	Concentration (μg/mL)
1	20, 18, 16, 14, 12, 10, 8, 6, 4, 2
2	2, 1.8, 1.6, 1.4, 1.2, 1.0, 0.8, 0.6, 0.4, 0.2
3	0.2, 0.18, 0.16, 0.14, 0.12, 0.10, 0.08, 0.06, 0.04, 0.02
4	0.02, 0.018, 0.016, 0.014, 0.012, 0.010, 0.008, 0.006, 0.004, 0.002, PBS

binding assays and inhibition assays. We used inhibition assays for these experiments. That is why the measured response was inversely related to the concentra- tion of analyte in the samples (18). The running buffer was used to dilute the samples. Experiments were repeated 5 times for each sample. The flow rate was 30 μL/min and the volume was 90 μL^3 (180 sec). Ten mM NaOH was used for the regeneration step, which separates antibody and antigen from each other for the next test.

Results and Discussion

Conditions of immobilization Preconcentration tests from pH 7.0 to 3.5 to select the optimum pH buffer were carried out. The results showed high resonance unit (RU) values from pH 4.5 to 3.5. Among these, pH 4.5 was selected due to the satisfactory characteristics of both the association and dissociation reactions. At pH 4.0 and 3.5, the association reactions were satisfactory however the dissociation reactions were unstable because the antibody residue on the surface of gold chip was not washed away (Fig. 4).

AFM image analysis Photograph of antibody immobilization using an AFM was taken. The carboxylated dextran matrix based (CM5) gold chip surface had an even distribution. The surface roughness (RMS), and mean height values of dextran on the gold chip were 10.03 and 42.47 Å (Fig. 5A). By comparison, the values for GST protein were 538.67 and 1,600 Å, which were larger than those of dextran (Table 2). The surface on which the antibody was immobilized had a sharp distinction compared with the areas without covalent linkages. The proteins were successfully immobilized on the surface of dextran (Fig. 5B).

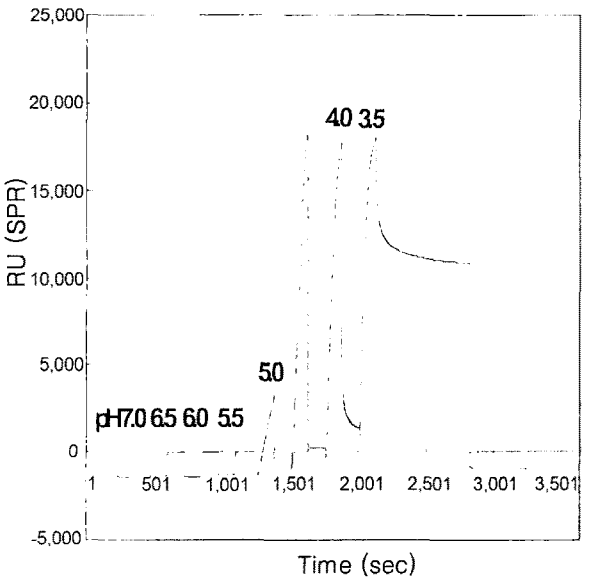

Fig. 4. Preconcentration test results at various pH values.

Association and dissociation reactions by concentration Association and dissociation reactions by dilution for carbamate pesticides (carbofuran, carbaryl, and benfuracarb) were tested. Typically, the time required for detection using HPLC and GC/MS is approximately 2 hr. In contrast, surface plasmon resonance equipment (Biacore 3000) was much faster with a 10.6 min reaction time, including regeneration (Fig. 6).

Sensitivity of SPR measurements To evaluate the sensitivity of the SPR measurements, we performed a series of inhibition assays with pesticide concentrations ranging from 20 to 0.002 μg/mL. The regression curves have a negative slope relative to increasing concentration. The coefficients of determination were from 0.96 to 0.98 (Fig. 7a). A second series of dilutions was performed with analyte concentrations between 0.1 and 0.002 μg/mL. For this set of data, the coefficient of determination for

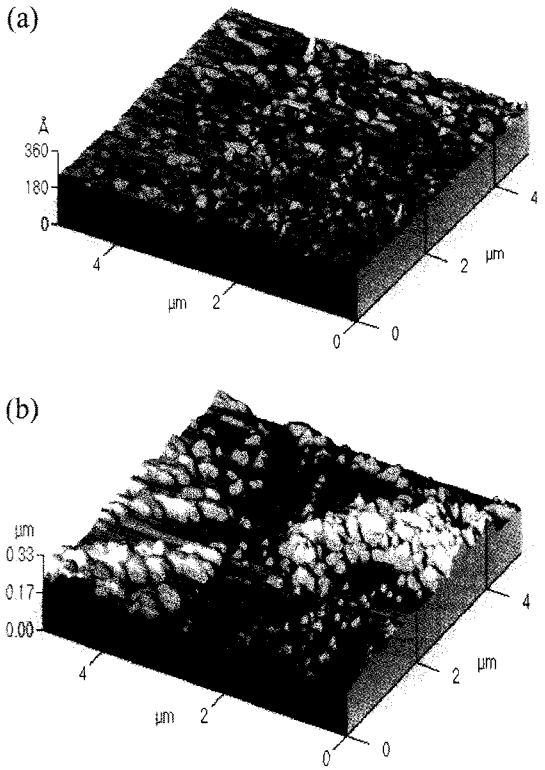

Fig. 5. Picture of gold chip surface spread with dextran and antibody; (a) dextran on the gold chip and (b) antibody on the dextran.

Table 2. Dextran and GST protein on the surface of CM5 sensor chips

	RMS (Å)	Mean height (Å)	Volume (m^3)
Dextran	10.03	42.47	0.11
GST	555	860	2.15

benfuracarb was 0.94, while it was 0.97 for the other analytes. We considered this to be the result of pipetting errors (Fig. 7b). Regressions and coefficients of determination for each analyte are described in Table 3. The sensitivity of the SPR device for detecting pesticide residues was 0.002 μg/mL, thus satisfying maximum residue limits (MRLs).

The Korea Food & Drug Administration (KFDA) sets MRLs for agricultural chemicals in agricultural produce, in particular for produce entering the food chain. These MRLs are set at levels that are unlikely to be exceeded if the agricultural chemicals are used in accordance with approved label instructions. At the time MRLs are set, the KFDA undertakes a dietary exposure evaluation to ensure that the levels do not pose an undue hazard to human health. Therefore, the gratification of MRLs is an important appraisal standard for the production of SPR biosensors.

The sensitivity, accuracy, and precision of the carbamate immunoassay tests were considered to be satisfactory for developing the biosensor. In the case of the benfuracarb analyte, although there was insufficient data due to dilution errors, the sensor would be suitable if better concentration standards were established.

For inhibition assays, the sensitivity was determined by the affinity of the interaction. To obtain a high sensitivity, it was advantageous to use a low concentration of the antibody. In practice, the sensitivity of the inhibition assay was close to 1 nM. An additional advantage of the inhibition format was that surfaces with small immobilized molecules were very stable and hundreds of samples could be analyzed on the sensor surface.

Reproducibility tests The average standard error and χ^2 for reproducibility tests are shown in Table 3. Each sample between 0.002 and 20 μg/mL was tested 5 times, making each dilution curve the result of over 200 tests (Table 1). As a result, the range of average standard errors and χ^2 values for each RU value is from 1.14 to 2.34 and from 0.17 to 0.35, respectively. The detection time was 684 sec (11.4 min), preparation time 110 sec, association time 180 sec, dissociation time 298 sec, and regeneration time 96 sec (Fig. 8). The fabricated protein GST maintained its activity for over 200 measurements.

However, this assay was not efficient for the simultaneous analysis of multiple samples since the Biacore 3000 machine has only a single line of flow and four flow cells. Each sample was applied after previous samples were analyzed and the cells were washed and regenerated. In contrast, the SPR assay could be very advantageous for the

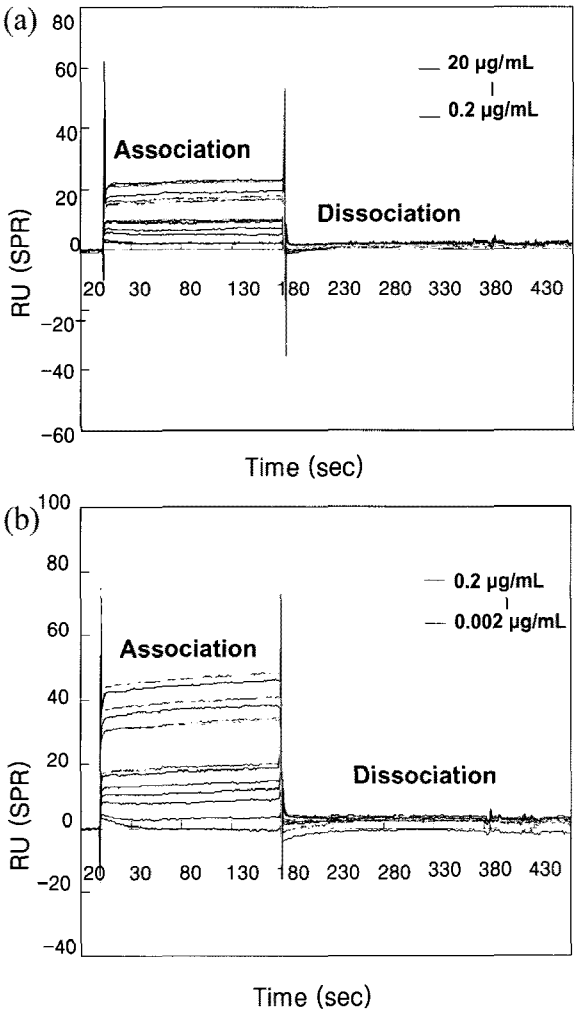

Fig. 6. SPR curves at various concentrations. (a) Sample 1, sample 2; (b) sample 3, sample 4.

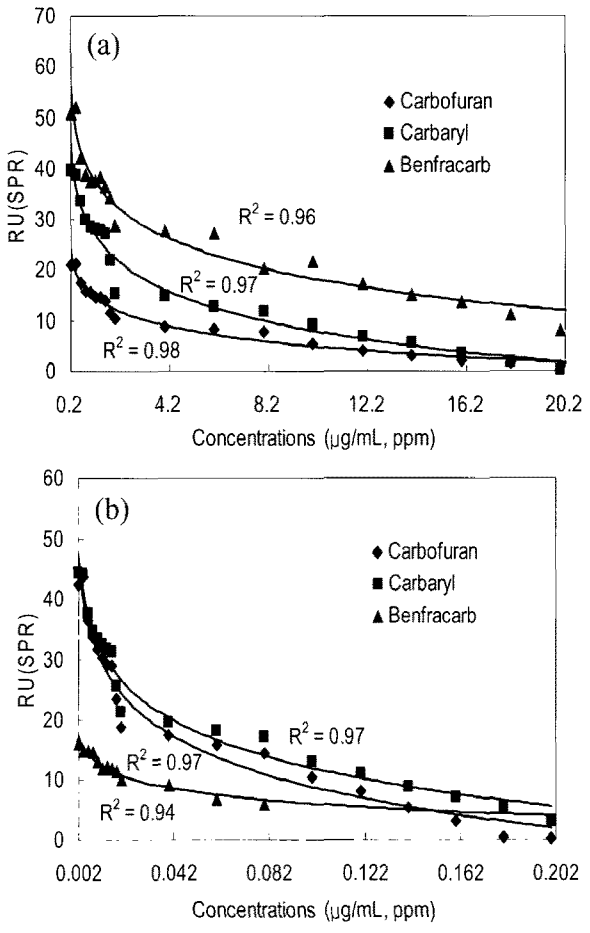

Fig. 7. Concentrations from 0.2 to 20 μg/mL (a) and from 0.002 to 0.2 μg/mL (b). Interaction between GST (26 kDa) and pesticides (Carbofuran, 221Da; Carbaryl, 201Da; and Benfuracarb, 411Da) plotted by concentration.

Table 3. Regressions of each composition

Pesticide	Concentrations (μg/mL)	Regression	Coefficient of determination	Average standard error	Average χ^2	MRL (μg/mL)
Carbofuran	20–0.2	y=-4.51 ln(x)+15.28	0.98	1.27	0.19	0.01
	0.2–0.002	y=-9.72 ln(x)–13.58	0.97	2.34	0.35	
Carbaryl	20–0.2	y=-8.85 ln(x)+28.33	0.97	2.07	0.31	0.20
	0.2–0.002	y=-9.13 ln(x)–9.09	0.97	2.27	0.34	
Benfuracarb	20–0.2	y=-9.06 ln(x)+39.11	0.96	1.14	0.17	0.22
	0.1–0.002	y=-2.90 ln(x)–0.67	0.94	1.20	0.18	

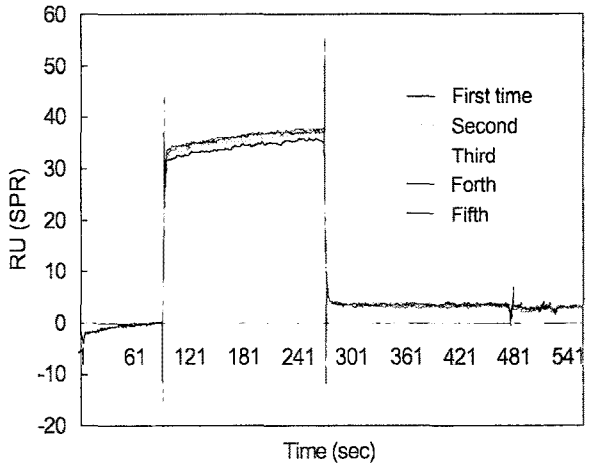

Fig. 8. Example of SPR curves for reproducibility tests performed 5 times with 0.01 μg/mL carbaryl.

detection of multiple kinds of pesticide residues if the SPR device has multiple flow cells. The SPR biosensors can detect the specific reversible binding of a reactant in solution to a binding partner immobilized on the surface of the sensor, and allow real-time detection and monitoring (19,20). A portable SPR biosensor to detect pesticide residues is possible if it has enough sensitivity and capacity.

Acknowledgments

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