Influence of Coating Ligands on Enzyme-linked Immunosorbent Assay of Toluene

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The specificity of conjugation site for coating ligands was investigated using toluene-bovine serum albumin (BSA) conjugates in which BSA was conjugated at the position of *o*-, *m*-, and *p*-toluic acid. Toluene-BSA conjugated at *p*-position showed a binding activity of about 89-95%, whereas, those conjugated at *o*- and *m*-position of toluene exhibited a binding activity of 5 and 11%, respectively. On the basis of the above result, coating ligands with various proteins (OVA, BSA, KLH) were compared by conjugating with *p*-toluic acid, and toluene-KLH was considered as the best coating ligand for this ELISA. Indirect competitive ELISA method was developed using anti-toluene antibody and *p*-position conjugated toluene-KLH. The dose-response curve constructed after kinetic and optimization studies showed a 1×10^{-4}–1×10^{2} mM detectable response range with 0.1 μM detectability. In specificity test of the antibody, the binding capabilities of aromatic compounds substituted with nitro-, alkyl-, chloro-, and hydroxyl group were larger rather than those of aromatic compounds (benzene, toluene and xylene) themselves. Also, tests with soil and water samples that had been spiked with toluene resulted in 102.7-113.7% recovery.

Keywords : Coating ligand, Competitive ELISA, Toluene, Benzene, Xylene.

Introduction

Environmental contamination by petroleum hydrocarbon become a serious social concern because of the frequent contamination of soil and ground water occurring at gasoline filling stations where it leaks from its storage place. Toluene is a clear, colorless liquid with a distinctive smell. It is produced as a by-product in the process of making gasoline, styrene and other fuels. Benzene also is a colorless and flammable liquid and it is used in various industries to make other chemicals and to manufacture rubber, dye, and so forth.[1] Benzene (B), toluene (T), including ethylbenzene (E) which constitutes approximately 2% of gasoline and xylene (dimehtyl benzene, X) have been used as solvents in printing. Gasoline[2] which contains from 12 to 54% aromatic compounds[3,4] is also used in the production of rubber and leather, and its exhaust constitutes over a half of amount in contaminated air.

People are exposed to the above materials through the skin or by drinking contaminated food or water, however, the main source of exposure is by inhaling contaminated air, especially in areas with heavy traffic and around gasoline filling stations. The leakage of BTEX from these sources to the soil and ground water brings about serious problems; BTX is known to be toxic and carcinogenic, and produces harmful effects on the nervous system. Several tests to determine the level of exposure measure BTEX and their breakdown products in blood or urine. Analytical methods such as gas chromatography require sophisticated instrument handling and a well-trained technical person to perform the analysis. Immunochemical analysis, however, is the most desirable method for a field screening test because, it provides the sensitivity and specificity to detect total levels of BTEX along with its related chemicals. Second, it can be adapted to various simple methods which can be performed in the field without having to transfer the sample from the location to the laboratory. These methods include an immunodetection strip, and an automatic mass screening method that utilizes specific recognition between the BTEX antigen and antibody.[5,6] Among the various immunochemical methods, ELISA is a basic tool used to evaluate antigen-antibody binding characteristics because it is simple to search the matching pairs of the antigen and its antibody. In developing ELISA to determine levels of BTEX, there are many factors to consider that affect assay sensitivity and specificity. For example, the preparation of the solid phase with coating antigen is the most important step because the solid phase serves as an anchor where all subsequent reactants will bind.[7] For this reason, toluene-protein conjugates, in which proteins were coupled at three positions (*o*-, *m*-, *p*-) of toluene, were synthesized as coating antigens. The effect of an antibody recognition ability was investigated in terms of the binding position of protein to be conjugated to the antigen. As mentioned above, optimizing the solid phase preparation was absolutely critical, and, thus, the functional abilities of five types of coating ligands were examined. On the basis of the results obtained, we developed an ELISA to determine levels of toluene. It was applied to determine levels of BTEX and closely related aromatic compounds

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substituted with nitro-, alkyl-, chloro-, and hydroxyl groups. This ELISA was also applied to the quantification of toluene-spiked samples of soil and water.

Experimental Section

Materials. Bovine serum albumin (BSA) used to prepare the buffer solution, *o*-phenylenediamine (OPD), *N*,*N*′-dicyclohexylcarbodiimide (DCC), coomassie blue G-250, dimethylformamide (DMF), and *N*-hydroxysuccinimide (NHS) were purchased from Sigma Chemical Co. (St. Louis, MO., USA). Peroxidase-conjugated sheep immunoglobulin G (IgG) fraction to rabbit IgG (whole molecule) were from Cappel (NC., USA). Rabbit anti-toluene IgG which was prepared using immunogen of toluene-bovine gamma globulin was purchased from Cortex Biochem (CA., USA). Bovine serum albumin (BSA), ovalbumin (OVA), keyhole limpet hemocyanine (KLH), and toluic acid for the synthesis of coating tracers were purchased from Pierce (IL., USA) and Fluka (Switzerland), respectively. Toluene used for standard was purchased from Kanto (Japan) and all chemicals used were of analytical grade. A microwell module (Maxisorp) was purchased from Nunc (Denmark) and an E_{max} precision microtiter plate reader (Molecular Devices Inc., CA., USA) was used to measure the optical density of ELISA results.

Preparation of toluene coating ligands. Coating ligands were prepared by applying the method in Yoon *et al.*[8,11] Briefly stated, 5 mg of toluic acid, NHS, and DCC were dissolved in 0.5 mL DMF and stirred slowly for 2 hours. The resulting NHS ester of toluic acid was dissolved in 0.5 mL of 0.2 M carbonate buffer, pH 8.8 containing 0.15 M KCl, and it was added to protein solution in PBS. After slightly mixing at 4 °C for 2 hours, 0.5 mL of 1 M NH_4Cl was added to stop the reaction. Toluene-BSA conjugate was pooled and dialyzed against 0.2 M carbonate buffer, pH 8.8.

Titration level assessment of antibody. Prior to obtaining the dose-response curve, the antibody concentration was measured yielding a response of OD 2.0 at 490 nm.[12] A microtiter plate was coated with 100 μL toluene-KLH coating ligand prepared in 50 mM carbonate buffer, pH 9.6 for 16 hours at 4 °C, and then washed three times with 250 μL PBST (PBS containing 0.05% tween).[20] The wells were blocked using 150 μL of 3% BSA in PBS for 30 minutes and washed three times. Microplate wells coated with toluene-KLH coating antigen were incubated for 90 min with 50 μL serially diluted antibody and 50 μL of 1% BSA in PBS. 100 μL of 1/2000 diluted enzyme tracer was added to each well and incubated for 90 min. After each incubation step, the plate wells were washed. After incubating for 10 min with OPD substrate, the color reaction of substrate was stopped by adding 50 μL of 4 N H_2SO_4. The optical density was read at 490 nm. The antibody concentration yielding a response of OD 2.0 at 490 nm was used as a titer level.

Dose-response curves for toluene. Based on the assessment of titer level of antibody, a dose-response curve for toluene was constructed. First, a microtiter plate was coated with 100 μL coating ligand in 50 mM carbonate buffer, pH 9.6 for 16 hours at 4°C and then washed three times with PBST. The wells were blocked with 150 μL of 3% BSA in PBS for 30 minutes, after washing, 50 μL free toluene and 50 μL toluene antibody were added to each well and incubated for two hours at room temperature. After a washing step, diluted peroxidase-conjugated sheep anti-rabbit IgG was added to each well and incubated for two hours. The color reaction of substrate for 15 minutes was stopped by adding 50 μL of 4 N H_2SO_4. The optical density was read at 490 nm and the standard curve was constructed by plotting the toluene concentration versus the relative response (%) to that at zero concentration of toluene.

Preparation of soil and water samples. 20 g Na_2SO_4 was added to 20 g soil and mixed to remove the moisture in soil. A fixed amount of toluene was added to the dry soil and then 40 mL methanol was added. The soil sample spiked with toluene was transferred to the ice bath and sonicated for 30 minutes. The sample was centrifuged to separate the methanol layer containing toluene. Toluene extracts were diluted to be in the detection range of the calibration curve constructed.

The water sample containing toluene were prepared. Stock solution of toluene was dissolved in a small volume of DMF and carefully mixed with buffer solution, because toluene was immiscible with water.

Results and Discussion

Three types of toluene-protein were synthesized by conjugating proteins at *o*-, *m*-, and *p*-position of toluene. They were challenged for the coating ligands on the

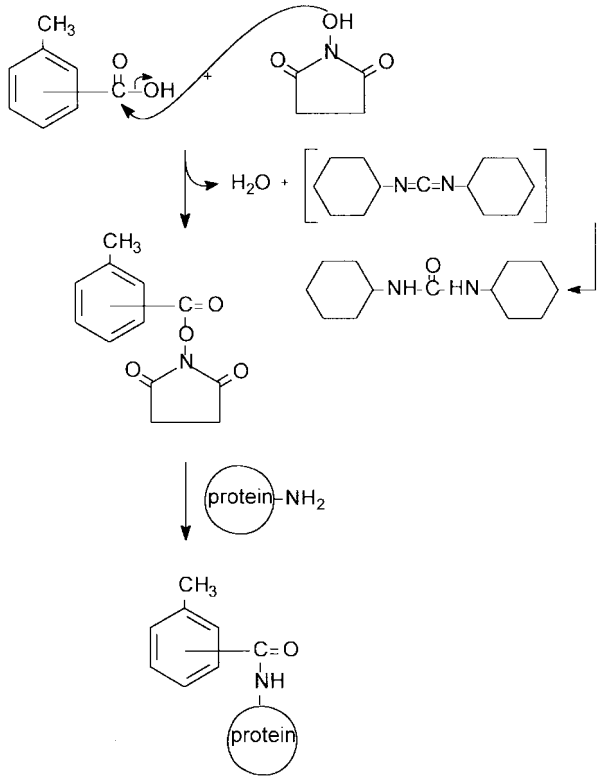

Figure 1. Preparation of toluene-protein conjugates for use as coating ligands. The coating ligands were synthesized by the reaction of toluic acid, NHS, and DCC with protein.

microtiter plate and competed with free toluene for toluene specific antibody. In the coupling between toluene and protein, we used toluic acid as an antigen, and BSA, OVA, and KLH as coupling proteins, respectively. NHS and DCC were applied as crosslinkers according to NHS ester method (Figure 1). The amount of proteins in toluene-protein conjugates were quantitated by the Bradford method instead of using UV absorbance values due to the overlapping of UV spectra at 280 nm between toluene and the protein.

We tested the capabilities of the antibody to recognize three different toluene-protein coating ligands in which the proteins had been conjugated at the *o*-, *m*-, and *ρ*-position of toluene. The OD values of (a), (b), and (c) in Figure 2 showed the degree of binding ability between the antibody and coating ligands on the well surface under the same reaction conditions. Their OD values were 0.186, 0.38, and 3.4, separately. When we converted the antibody binding ability to % binding capability, coating tracers made of BSA which was conjugated at the *o*-, *m*-, and *ρ*-position of toluene showed approximately 5, 11, and 100% binding activity. As seen in Figure 2, the toluene-BSA coating tracer, in which BSA was conjugated at *ρ*-position of toluene, had a binding ability about 10 to 20 times higher than those conjugated at the *o*-, and *m*-position of toluene. From the above results, we could assume that toluene-BSA that had BSA conjugated at *ρ*-position of toluene had very low steric hindrance to reacting with antibody, which resulted in improved assay sensitivity. On the other hand, coating ligands in which the BSA conjugated at the *o*- or *m*-position of toluene structurally hindered in the binding between the antigen and antibody. This indicates that the coupling position of the protein to the antigen is an important consideration when developing an ELISA, because it may affect the degree of exposure of the antigen epitope on the surface. Also, selection of coating ligand with a matching antibody is essential to develop the immunoanalysis method.

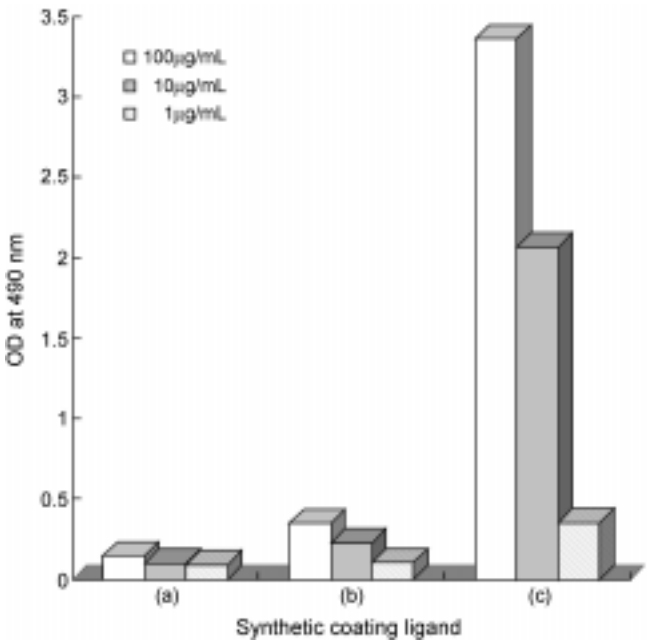

Figure 2. Comparison of rabbit anti-toluene IgG binding capacities with three types of coating ligands. Toluene-BSA of which BSA was conjugated at the *o*-position of toluene (a), at the *m*-position of toluene (b), at the *ρ*-position of toluene (c).

From these results, three toluene coating antigens were synthesized using different proteins: BSA, OVA, and KLH at the *ρ*-position of toluene. When antibody binding activity was evaluated after coating with the three conjugates, a matched pair of toluene-KLH and antibody had a binding efficiency that was about 2.2 to 3.3 times higher than that of toluene-BSA or toluene-OVA (toluene-KLH > toluene-BSA > toluene-OVA). We could observe that as molecular weight of the conjugated protein increased, the binding activity also increased. On the basis of above results, toluene-KLH, in which KLH conjugated at the *ρ*-position, was selected as the most appropriate coating antigen.

Assay optimization is essential prior to constructing the calibration curve,[12] so an optimization study was employed in every reaction step including the immobilization of the coating antigen, a competitive binding reaction of free analyte and the coated antigen for the antibody, and the binding between the antibody and a second antibody-enzyme conjugate.

In the kinetic study of toluene-KLH immobilization on the solid phase, maximum absorption was reached after 12 hours, and the coating level depended on the incubation temperature and coating ligand concentration. when we compared the coating efficiency of toluene-KLH incubated at 4 ^{o}C and RT, that of toluene-KLH incubated at RT was approximately 2.7-13% higher than that incubated at 4 ^{o}C.

The competitive binding between free toluene and toluene-protein on the solid phase for antibody required 1.5 hours for a replacement that was over 85% of the maximum, and the binding of a second antibody-enzyme tracer with the toluene antibody took 1 hour for a maximum binding of over 90%. In the concentration study of toluene-KLH, the saturation of one well surface was reached at 40 μg/mL or higher.

The antibody titer level was assessed by measuring the amount of antibody needed to yield OD 2.0 at 490 nm. When 0.1, 0.5, 1, 10, 20, and 40 μg/mL toluene-KLH were coated on the wells, antibody titer levels were 90, 10.1, 3.9, 0.3, 0.21, and 0.16 μg/mL, respectively. From the above results, we confirmed that the antibody titer level decreased as the amount of coating ligand increased.

From a series of coating ligand dilution studies,[13] dose-response curves with high sensitivities were obtained using 1 and 10 μg/mL toluene-KLH. A dilution study of a second antibody-enzyme tracer using a fixed amount of coating ligand and antibody exhibited no change in the detection range or sensitivity.

As shown in Figure 3, a typical dose-response curve with the best sensitivity was established using 1 μg/mL toluene-KLH coating ligand and 1/4000 diluted HRP conjugated sheep anti-rabbit IgG. Detectability in this curve was determined to be 0.1 μM with 0.23 mM of IC_{50} which is a concentration of 50% displacement in competitive ELISA. The coefficient of variation (CV) was 4.8% using 1.085 mM free toluene concentration and its detectable response range was 1×

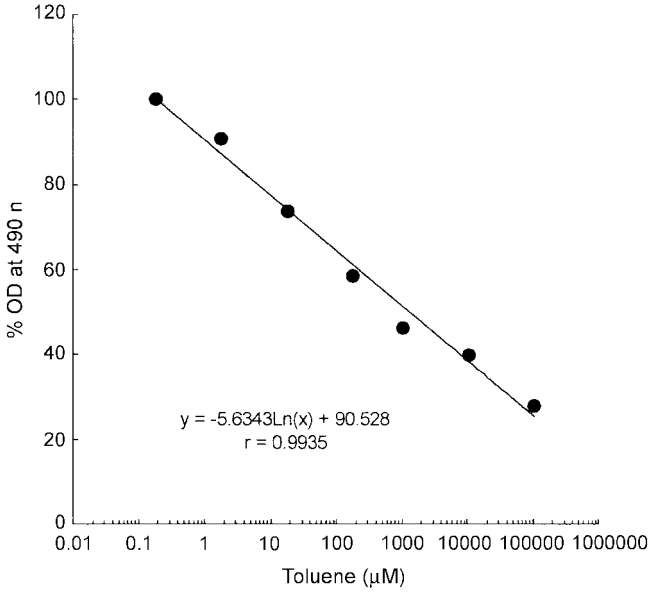

Figure 3. Toluene dose-response curve by ELISA It was constructed with 1 g/mL toluene-KLH and 1/4000 diluted peroxidase-conjugated sheep anti-rabbit IgG tracer.

10^{-4}–1×10^{2} mM.

The specificity of the antibody was tested with aromatic petroleum hydrocarbons that had chemical structures similar to toluene.[7] Cross-reactivity could be expressed as the ratio of the 50% binding affinity for toluene to the binding affinity for cross-reactants that were assayed under identical assay conditions. Cross-reactants were divided into four groups; toluene, benzene, phenol substituents, and BTEX including their mixtures.

Table 1. Cross-reactivities of various toluene analogues with toluene antibody

Compound	ELISA	
	IC_{50}[a] (mM)	Cross-reactivity[b] (%)
Toluene	12	100
2-Ethyltoluene	1.3	923
2-Nitrotoluene	0.080	15,000
4-Chlorotoluene	2.2	545
Benzene	30	40
Ethylbenzene	0.012	100,000
Nitrobenzene	0.033	36,000
1,2-Dichlorobenzene	0.11	11,000
Chlorobenzene	2.5	480
Phenol	3.2	375
2-Methylphenol	1.7	706
Nitrophenol	1.3	923
2-Chlorophenol	0.040	30,000
σ-Xylene	14	86
m-Xylene	10	120
ρ-Xylene	10	120
BTX[c]	13	92

[a]IC_{50} means a concentration of 50% displacement in competitive ELISA. [b]Percent cross-reactivity defined as (toluene concentration for 50% displacememt)/(toluene analogue concentration for 50% displacement) × 100. [c]Benzene : toluene : total xylene = 1 : 1 : 1 (v/v)

Table 1 summarized the relative cross-reactivity of various compounds and their mixtures compared to the reactivity of toluene. Unexpectedly, toluene antibody reactivity was much stronger with related aromatic compounds substituted with nitro-, alkyl-, chloro-, and hydroxyl group than those with benzene, toluene, *o*-, *m*-, ρ-xylene, and their mixtures. Cross-reactivities (%) of benzene, toluene and *o*-, *m*-, ρ-xylene were 40, 100, 86, 120, and 120%, and that of a mixed sample which was prepared by adding benzene, toluene and xylene in 1 : 1 : 1 volume ratio was 92%. Ethylbenzene was the most reactive chemical with this antibody. The response between ethylbenzene and the toluene antibody was three orders higher than that of toluene.

To evaluate the ELISA that had been developed, the amount of toluene spiked in distilled water or soil samples was quantitated. Toluene-spiked water samples were prepared by dissolving toluene in a small volume of DMF, and diluting with water because of the immiscibility of toluene with water. Prepared soil samples were extracted with methanol and diluted with buffer. Prior to employing toluene ELISA with soil samples, we evaluated the effect of methanol used as an extract solution on an immunoreactivity. The specific binding ability between toluene and antibody was maintained in a matrix which contained 5% or less methanol, whereas, the antigen-antibody interaction decreased rapidly in a matrix containing more than 5% methanol (Figure 4). Thus, toluene extracted by methanol was diluted 20 times or more by assay buffer to minimize the matrix effect. Figure 5 shows the results of the recovery test employed with soil (Figure 5A) and water samples (Figure 5B) determined by ELISA. As seen in Figure 3, the range in the standard curve was very broad (0.2 μM-100 mM) and it was easy to assess toluene concentration in the samples.

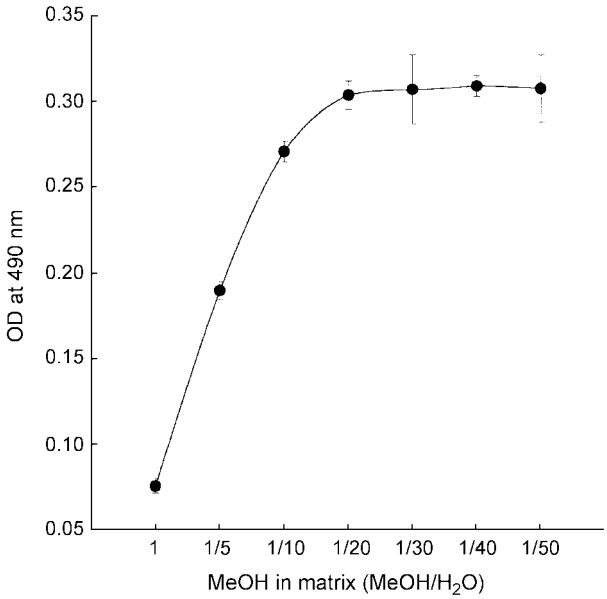

Figure 4. Matrix effect of methanol in soil extract. Soil was extracted with methanol prior to application in ELISA.

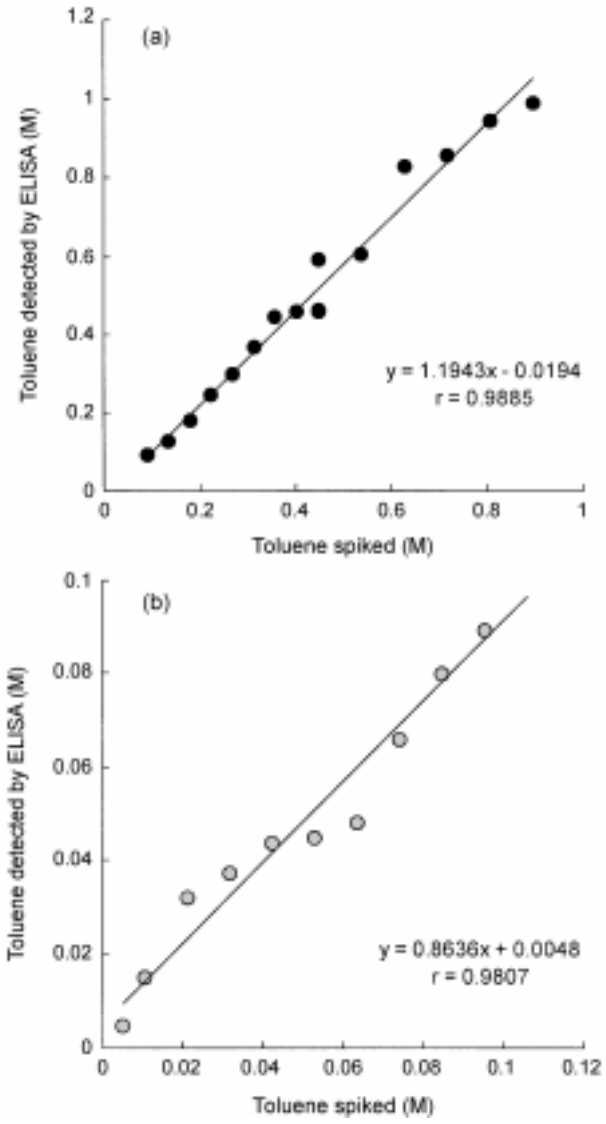

Figure 5. Recovery test for toluene in soil and water samples. (a) Correlation between the toluene level spiked in soil (x) and the toluene level determined by ELISA (y); $n = 15$ (b) Correlation between the toluene level spiked in water (x) and the toluene level determine by ELISA (y); $n = 10$.

In summary, this study has shown that the coating ligand used as a competitor of free analyte in an indirect competitive ELISA of toluene could significantly influence the assay performance. Therefore, careful investigation of hapten derivatization was required to detemine the best coupling position of the carrier protein. Toluene-KLH, in which KLH was coupled at the ρ-position of toluene, showed a better binding efficiency than that obtained from toluene-KLH, in which KLH was coupled at the *o*- or *m*-position of toluene. With this ligand, we obtained a toluene calibration curve with a detection range of 0.0001-100 mM and 0.1 μM detectability.

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