

Hapten Synthesis and Influence of Coating Ligands on Enzyme-linked Immunoreaction of DDT

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For the development of immunodetection method of 4,4'-dichlorodiphenyl-2,2,2-trichloroethane (*p,p'*-DDT), a persistent and broad toxic organochlorine insecticide, various DDT derivatives were synthesized and characterized for the use of immunogens and the coating ligands for the antibody evaluation. The appropriate lengths of linkers were introduced to investigate more efficient DDT derivatives. Among these hapten derivatives, 2,2-Bis(4-chlorophenyl)acetic acid (DDA), 5,5-Bis(4-chlorophenyl)-5-hydroxypentanoic acid (DDHP) and 5,5-Bis(4-chlorophenyl)-5-chloropentanoic acid (DDCP) were conjugated with keyhole limpet hemocyanin (KLH) for the use of immunogen to produce antibodies. 6,6-Bis(4-chlorophenyl)-6-hydroxyhexanoic acid (DDHH) and 3-[6,6-Bis(4-chlorophenyl)-6-hydroxyhexanoylamino]propanoic acid (DDHHAP) in addition to above hapten derivatives were conjugated to ovalbumin (OVA) and bovine serum albumin (BSA) for the use of coating ligands to measure the titration level of antibody and the displacement of free analytes. Three matching pairs of antibodies and coating ligands were selected for the simultaneous detection of *p,p'*-DDT and its related compounds of DDA and 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (*p,p'*-DDE) by investigating the displacement of free analytes in an indirect ELISA. These were PAb #1 and coating ligand DDCP-OVA, PAb #1 and DDHHAP-OVA, and PAb #3 and DDHHAP-OVA. The most useful immunoreaction for DDT analytes were obtained using PAb #3 and coating ligand DDHHAP-OVA showing 3.4 ng/mL of lower limit of detection. These results indicated that titration level and free analytes displacement were greatly influenced by hapten derivatized and carrier proteins conjugated.

Key Words : Immunoreaction, DDT, Coating ligand, Polyclonal antibody

Introduction

DDT has been used since the 1940s in Africa and elsewhere to prevent the spread of malaria, and for works by repelling or killing the mosquitoes that carry the virus.¹⁻³ It is an organochlorine pesticide used widely in agriculture and a broadly toxic compound that is highly stable and insoluble in water and soluble in organic solvent. They tend to pass with food fats into the body and to accumulate in fat deposit in animal tissue, as well as in the environment. Technical grade DDT is a mixture of three forms, *p,p'*-DDT (85%), *o,p'*-DDT (15%), and *o,o'*-DDT (trace amounts). DDT is slowly degraded in tissues mainly to 2,2-Bis(4-chlorophenyl)acetic acid (DDA) and its two major decomposition products are 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (DDE)⁴ and 2,2-bis(4-chlorophenyl)-1,1-dichloroethane (DDD).⁵ Urinary excretion of DDA has been reported as a sensitive marker of DDT exposure in humans and other mammals.^{6,7} The urinary excretion of DDA for normal and healthy male ranged from 0.025 to 0.120 $\mu\text{g/mL}$ with a mean of 0.066 $\mu\text{g/mL}$ and falls in lines with those reported earlier.⁸⁻¹⁰ DDD was also used to kill pests, and one form of DDD was used medically to treat cancer of the adrenal gland. Animal studies show that DDT and its metabolites affect the nervous system.¹¹ However, tests in animal suggest that exposure to DDT may have a harmful effect on reproduction, and long-term exposure may affect the liver.

Consequently, the use of DDT was totally banned in developed countries in the 1970s. DDT or its breakdown products are still found in air, water, and soil samples.¹²⁻¹⁴ The common procedure for the analysis of DDT and its metabolites are GC/MS,^{15,16} but the method requires extensive sample preparation and clean up. Enzyme-linked immunosorbent assay (ELISA) is suitable for environmental residue analysis¹⁷ due to its high sensitivity, small sample volume requirements, cost-effectiveness and speed.

The important components of ELISA are the antibody¹⁸ and coating ligand, since antibody is responsible for the sensitive and specific recognition of the analyte, and coating ligand have to compete on the affinity between antibody and analyte. Therefore, hapten design is a key step in the development of competitive ELISA for the use as immunogen and coating ligand. For further assay improvement, the synthesis of haptens with different spacer arms attached through different molecule is generally recommended.¹⁹⁻²¹

This paper describes the synthesis of several haptens and their affinity to antibodies. The screening of the antibodies for sensitive detection is presented, and the influence of different haptens and carrier proteins are evaluated for the use as coating ligand.

Experimental Section

Materials. N-Hydroxysuccinimide (NHS), dimethylform-

amide (DMF), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), bovine serum albumin (BSA), ovalbumin (OVA), precoated preparative TLC plates (Art. 13895, PSC-Fertigplatten Kieselgel 60 F254 for preparative chromatography, 20 × 20 cm, 1mm) were purchased from Merck Co. (Germany). Buffers were 10 mM phosphate buffered saline (PBS), pH 7.2 and 50 mM carbonate buffer, pH 9.6. ELISA substrate contained 70 mM *o*-phenylenediamine (OPD) and 5.1 mM hydrogen peroxide in 53 mM sodium citrate buffer containing 10 mM sodium hydrogen phosphate, pH 5.3. PBST, pH 7.2 contained 10 mM PBS with 0.05% Tween 20. All chemicals were used of analytical grade, and the solutions were made in deionized water using the Milli-Q water purification system (Millipore Inc., MA, USA). A microwell module (maxisorp) was purchased from Nunc (Denmark) and an Emax precision microtiter plate reader (Molecular Devices Inc., CA, USA) was used to measure the optical density of the ELISA results.

Preparation of DDT derivatives. *2,2-Bis(4-chlorophenyl)-acetic acid (DDA)*: DDA was prepared from *p,p'*-DDT by the method of Grummitt *et al.*²²: ¹H NMR (300 MHz, CDCl₃) δ 7.31 (d, *J* = 8.58 Hz, 4H, ArH), 7.23 (d, *J* = 8.52 Hz, 4H, ArH), 4.99 (s, 1H, ArCH).

5,5-Bis(4-chlorophenyl)-5-hydroxypentanoic acid (DDHP): DDHP was prepared by applying the method of Abad *et al.*²⁰ Briefly, a solution of glutaric anhydride (2 g, 17.5 mmol) in dry THF in the presence of nitrogen was stirred at 40 °C until the anhydride dissolved. 4-Chlorophenylmagnesium bromide (70.0 mmol in 1 M ether solution) was added dropwise. The reaction mixture after being stirred at the same temperature for 3 hours was allowed to cool to room temperature and stirred for 14 hours. The reaction mixture was poured over a saturated solution of ammonium chloride (30 mL). The aqueous layer was washed with ether, acidified with 1 M HCl. The insoluble material was filtered and the aqueous layer was extracted with ether (2 × 30 mL). The organic layer was washed with water and dried over MgSO₄. After solvent removal, chromatographic purification (ethyl acetate : *n*-hexane, 1 : 5) allowed to produce DDHP (0.41 g, 14%) as oily yellowish compound: ¹H NMR (300 MHz, CD₃OD) δ 7.31 (m, 8H, ArH), 2.40 (t, *J* = 6.93, 2H, C₁₁-CO₂H), 2.28 (m, 2H, C(OH)C₁₂), 1.61 (m, 2H, CH₂C₁₂CH₂).

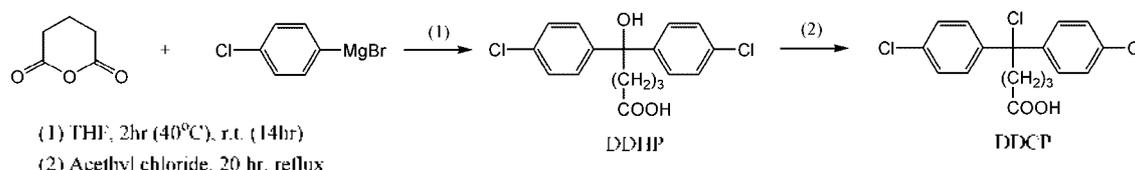
5,5-Bis(4-chlorophenyl)-5-chloropentanoic acid (DDCP): A solution of 5,5-bis(4-chlorophenyl)-5-hydroxypentanoic acid (0.1 g, 0.29 mmol) in acetyl chloride (3 mL) was stirred under reflux for 20 hours and solvent was removed. Purification by prep thin layer chromatography (ethyl acetate : *n*-hexane, 1 : 5) produced DDCP (0.069 g, 66%) as a hygroscopic solid: ¹H NMR (300 MHz, CD₃OD) δ 7.31 (m, 8H,

ArH), 2.40 (t, *J* = 6.93, 2H, C₁₁-CO₂H), 2.35 (m, 2H, C(OH)C₁₂), 1.61 (m, 2H, CH₂C₁₂CH₂); GC/MS (*m/z*): 358 (M+2).

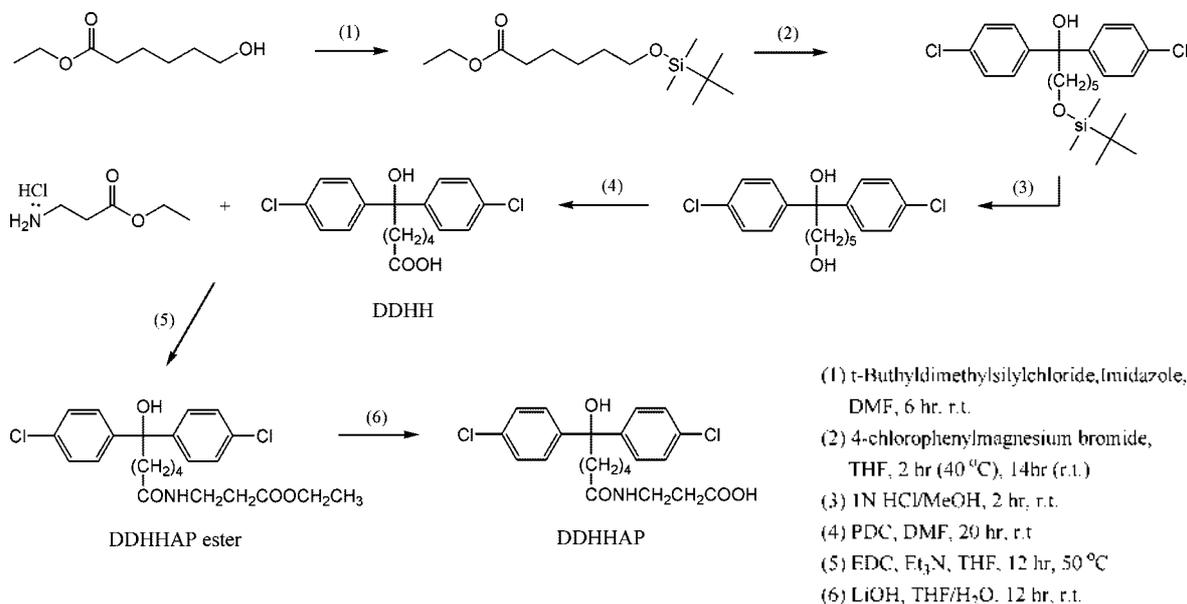
6,6-Bis(4-chlorophenyl)-6-hydroxyhexanoic acid (DDHH): A solution of ethyl-6-hydroxyhexanoate (1.0 g, 6.05 mmol) and imidazole (0.5 g, 7.27 mmol) in DMF (10 mL) after being stirred at room temperature for 20 minutes was allowed to cool to 0 °C. *tert*-Butyldimethylsilyl chloride (1.0 g, 6.44 mmol) in DMF (15 mL) was added to this solution. The reaction mixture was stirred at room temperature for 6 hours and quenched the reaction with water (1 mL). The solvents were evaporated *in vacuo* and the residue was partitioned between ethyl acetate (3 × 50 mL) and water (100 mL). The organic layer dried over MgSO₄ and concentrated to produce an oily colorless compound 1 (1.3 g, 76%). [Ethyl 6-(1,1,2,2-tetramethyl-1-silapropoxy)hexanoate]: ¹H NMR (300 MHz, CDCl₃) δ 4.21 (q, *J* = 7.14, 2H, CO₂C₁₁), 3.60 (t, *J* = 6.41, 2H, SiOC₁₁), 2.30 (t, *J* = 7.52, 2H, COC₁₁), 1.60 (m, 2H, SiOCH₂C₁₁), 1.51 (m, 2H, COCH₂C₁₁), 1.36 (m, 2H, SiOCH₂CH₂C₁₁), 1.26 (t, *J* = 7.11, 3H, CO₂CH₂C₁₁), 0.90 (s, 9H, C(C₁₁)₃), 0.05 (s, 6H, Si(C₁₁)₂).

The compound 1 (1.0 g, 3.64 mmol) was placed in dry THF (40 mL) in the presence of nitrogen. After warming to 40 °C, the reaction mixture was added 4-chlorophenylmagnesium bromide (10.9 mmol of a 1 M ether solution) over a period of 30–40 minutes and allowed to stir for 2 hours. The reaction mixture finally was stirred at room temperature for 15 hours and poured over a saturated solution of ammonium chloride (20 mL). The aqueous layer was washed with ether (2 × 20 mL), acidified with 1 M HCl, and extracted with ether (2 × 20 mL). The organic layer was washed with water, dried over MgSO₄ and concentrated. The product was purified by flash chromatography (ethyl acetate : *n*-hexane, 1 : 10) and the compound 2 (0.95 g, 49%) produced as yellow syrup compound. [1,1-Bis(4-chlorophenyl)-6-(1,1,2,2-tetramethyl-1-silapropoxy)hexan-1-ol]: ¹H NMR (300 MHz, CDCl₃) δ 7.33 (d, *J* = 9.03, 4H, ArH), 7.27 (d, *J* = 8.94, ArH), 3.56 (t, *J* = 6.30, 2H, SiOC₁₁), 2.22 (m, 2H, C(OH)C₁₁), 1.47 (m, 2H, SiOCH₂C₁₁), 1.36 (m, 2H, HOCH₂C₁₁), 1.26 (m, 2H, SiOCH₂CH₂C₁₁), 0.88 (s, 9H, C(C₁₁)₃), 0.03 (s, 6H, Si(C₁₁)₂).

A solution of compound 2 (0.50 g, 1.10 mmol) in methanol (3 mL) was added with 2% HCl solution and the mixture was stirred at room temperature for 2 hours. The reaction mixture was extracted with chloroform (3 × 10 mL) and the organic layer was washed with water and dried over MgSO₄. The solvent concentrated to produce compound 3 (0.75 g, 100%) as a white solid. [1,1-Bis(4-chlorophenyl)-hexane-1,6-diol]: ¹H NMR (300 MHz, CD₃OD) δ 7.38 (d, *J*



Scheme 1. Synthesis of haptens DDHP and DDCP.



Scheme 2. Synthesis of haptens DDHII and DDHIIAP.

= 8.67, 4H, Ar \underline{H}) 7.26 (d, J = 8.67, Ar \underline{H}) 3.49 (t, J = 6.49, 2H, CH₂C \underline{H}_2 OH) 2.24 (m, 2H, HOCC \underline{H}_2) 1.47 (m, 2H, HOCH₂C \underline{H}_2) 1.32 (m, 4H, HOCH₂CH₂C \underline{H}_2 C \underline{H}_2).

A solution of compound 3 (0.5 g, 1.47 mmol) and PDC (1.70 g, 4.43 mmol) in DMF (12 mL) was stirred at room temperature for 15 hours and the solvent was evaporated *in vacuo*. The crude mixture was dissolved in enough dichloromethane and the insoluble solids were filtered. The filtrate was removed and the resulting residue was purified by flash chromatography (chloroform : methanol, 15 : 1). It produced the DDHH (0.42 g, 63%) as a white solid: ¹H NMR (300 MHz, CD₃OD) δ 7.39 (d, J = 8.61, 4H, Ar \underline{H}) 7.26 (d, J = 8.58, Ar \underline{H}) 2.24 (m, 2H, HOCC \underline{H}_2) 1.61 (m, 2H, COC \underline{H}_2) 1.30 (m, 4H, COCH₂C \underline{H}_2 C \underline{H}_2).

3-[6,6-Bis(4-chlorophenyl)-6-hydroxyhexanoylamino]propanoic acid (DDHIIAP): Et₃N (0.1 mL) was added to neutralize a solution of β -Alanine ethyl ester hydrochloride (0.043 g, 0.28 mmol) in THF (3 mL) at room temperature for 10 minutes. Then, EDC (0.065 g, 0.33 mmol) and 6,6-Bis(4-chlorophenyl)-6-hydroxyhexanoic acid (0.1 g, 0.28 mmol) were added to a solution of neutralized amine at 0 °C. The reaction mixture was stirred at 50 °C for 10 hours and the solvent was evaporated. The residue was dissolved in CHCl₃, washed with 0.5 N HCl, water and dried over MgSO₄. After rotary evaporation, the product was purified by column chromatography (ethyl acetate : *n*-hexane, 1 : 10). This ester 4 (0.095 g, 74%) was produce as syrup pale yellow compound. This ester 4 (0.050 g, 0.11 mmol) and LiOH in THF/H₂O (3 mL) were stirred at room temperature for 12 hours.^{23,24} After acidify, the reaction mixture was extracted with 10% MeOH/CHCl₃ (5 \times 5 mL), and dried over MgSO₄. The solvent concentrated to produce DDHIIAP (0.034 g, 72%) as a yellow syrup compound: ¹H NMR (300 MHz, CD₃OD) δ 9.32 (brt, 1H, CON \underline{H}) 7.32 (d, J = 8.73, 4H, Ar \underline{H}) 7.25 (d, J = 8.52, 4H, Ar \underline{H}) 3.27 (m, 2H,

CONHC \underline{H}_2) 2.57 (t, J = 7.24, 2H, CONHCH₂C \underline{H}_2) 2.24 (m, 4H, C \underline{H}_2 CONH, C(OH)C \underline{H}_2) 1.72 (m, 4H, C(OH)CH₂C \underline{H}_2 , C(OH)CH₂CH₂C \underline{H}_2).

Preparation of assay standards. PBS (10 mM, pH 7.2) was used as working buffer for all enzyme-linked immunosorbent assay (ELISA) experiments. A stock solution of 1 mg/mL DDT in DMSO was serially diluted in PBS to 0.1 ng/mL. The same method was used to prepare cross-reactant standards at concentrations of 0.1, 1, 10, 100, 1000, 10000 ng/mL. Standards and cross-reactants were stored at 4 °C.

Preparation of immunogens. Immunogens to produce DDT antibodies were prepared by the following method. 2,2-Bis(4-chlorophenyl)acetic acid (DDA) 2.0 mg (7.12 μ mol) and EDC 1.6 mg (8.18 μ mol) were dissolved in 1 mL DMF and stirred at room temperature for 30 minutes. A solution of KLH (10 mg) in 4 mL of 50 mM carbonate buffer (pH 9.6, containing 0.15 M KCl) was added the reaction mixture over a period of 30 minutes and allowed to stir at room temperature for 6 hours. The reaction mixture after being stirred at 4 °C for 6 hours was allowed to remove the insoluble solids by centrifuge. DDA-KLH conjugate was pool and dialyzed with PBS at 4 °C, and confirmed spectrophotometrically. UV-vis spectra showed qualitative differences between carrier protein and conjugate at the region of maximum absorbance of hapten. The molar ratio of hapten to carrier protein in conjugate was then estimated from the spectral data of hapten, carrier protein, and the corresponding conjugate. The DDHP-KLH and DDCP-KLH were prepared in the same way as DDA-KLH using DDHP and DDCP in place of DDA.

Preparation of coating ligands. Combination of five haptens (DDA, DDHP, DDCP, DDHH, DDHIIAP) and two carrier proteins (BSA, OVA) were used for the coating ligands. They were prepared by the following method. Hapten was covalently attached to a carrier protein using the

modified active ester method (Langone and Van Vunakis, 1982).²⁵ DDA 3.14 mg (11.2 μmol), NHS (2 mg, 16.9 μmol) and EDC (3.3 mg, 16.9 μmol) were dissolved in 0.5 mL DMF and stirred slowly for 2 hours. A solution of BSA (10 mg) in 4 mL of 50 mM carbonate buffer, pH 9.6, containing 0.15 M KCl was added the resulting supernatant. After slightly mixing at room temperature for 4 hours and standing overnight at 4 °C, the insoluble solids was removed by centrifuge. DDA-BSA conjugate was pooled and dialyzed with PBS at 4 °C, and profiled by monitoring at a wavelength of 280 nm. The other hapten-protein conjugates were prepared in the same way. Five BSA conjugates and five OVA conjugates were prepared using five DDT derivatives (DDA, DDHP, DDCP, DDHH, DDHHAP).

Preparation of DDT antiserum. DDT antisera were developed in rabbit using each DDA-KLH, DDHP-KLH and DDCP-KLH immunogen, initially immunizing with complete Freund's adjuvant (CFA) emulsion, followed by twice boost injection with incomplete Freund's adjuvant (IFA) emulsion for 3 weeks interval. Three kinds of antisera, PAb #1, PAb #2 and PAb #3, were obtained from third bleeding of rabbit.

Assessment of titration levels of the antibodies by ELISA. A microtiter plate was coated with 50 μL (10 $\mu\text{g}/\text{mL}$) of DDA-BSA coating ligand in 50 mM carbonate buffer (pH 9.6), for 12 hours at 4 °C, and then washed three times with 200 μL PBST. The wells were blocked using 150 μL of 1% BSA in PBS for 2 hours at room temperature and washed three times. The DDA-BSA coated microwells were incubated for 2 hours with 50 μL of serially diluted antibody in PBS-1% BSA solution at room temperature, and washed. HRP-conjugated rabbit- α -IgG (50 μL of 1/1000 diluted solution) was added to each well and incubated for 2 hours at room temperature, and then washed four times with PBS-1% BSA. After incubating for 10 minutes with OPD substrate, the color reaction of enzyme substrate was stopped with 50 μL of 2 N H_2SO_4 . The optical density was read at 490 nm. The titer level of antisera was chosen by the level of antibody dilution to produce approximately 70% of maximum absorbance. The titer levels of antisera for the other coating ligands were obtained in the same way.

Dose-response curve for DDT analytes by ELISA. A dose-response curve for each DDT analyte (DDT, DDA, DDE) was established based on the assessment of titer level of antibody. Antibody coating step was same as one in the antibody titration method. To construct a dose-response curve of DDT analytes, 60 μL free DDT analyte standard and 60 μL antibody were mixed and pre-incubated for 2 hours at room temperature. Aliquots (100 μL) of the resulting mixture was added to the hapten-protein coated wells. The remaining steps were then performed as described above. All assays were run in duplicate.

Results and Discussion

An appropriate hapten design should preserve resembling DDT chemical structure and modified structures were produced as a consequence of spacer arm attachment. The

successful production of anti-hapten antibodies is determined to predict the location of certain hapten in the three-dimensional structure of the carrier protein. Carboxy or amino-terminal regions are often exposed. Five kinds of haptens were synthesized by deriving carboxyl group into DDT derivatives because the molecular structure of DDT is clear that it cannot be directly coupled to proteins, since they lack a convenient group for this reaction. DDA, DDHP and DDHH were synthesized by replacing CCl_3 group in DDT as spacer arm through the carbon atom that joins the two aromatic rings by C-0, C-3 and C-4 carboxylic acid. DDCP was synthesized by replacing DDHP hydroxy group of the carbon atom by chlorine. Also DDHHAP designed to include amide bond with long spacer arm of the hapten. It was prepared by hydrolyzing DDHP and coupling β -alanine ethyl ester. $^1\text{H-NMR}$ spectrum of each DDT hapten was determined to confirm the structure of derivatization.

Consistent with their highest similarity to DDT haptens, DDA, DDHP and DDCP that were coupled with KLH were used for immunizing rabbits. DDT hapten-protein conjugates, that were combination of five haptens (DDA, DDHP, DDCP, DDHH, DDHHAP) and two carrier proteins (BSA, OVA), were used for coating ligands. The amount of proteins in hapten-protein conjugates were quantitatively determined by the Bradford protein measurement method instead of using UV absorbance values due to the overlapping of UV spectra at 280 nm between hapten and protein. BSA has 59 lysine (30-35 are available for coupling) and OVA has 20 lysine. Since lysine has side chain amino group, it can couple to carboxy group of hapten.

Three kinds of polyclonal antibodies (PAb) obtained from the hapten-KLH conjugates were characterized for the affinity and specificity to DDT analytes with ten coating ligands. The absorbance values in figure 1(A) showed the degree of binding ability between the antibodies and BSA conjugated coating ligands on the well surface under the same reaction procedures. Higher binding affinities for all three antibodies were observed with the coating ligand of longer spacer arms, such as DDHH-BSA and DDHHAP-BSA. OVA-conjugated coating ligand showed different pattern than BSA-conjugated coating ligands (Figure 1(B)). DDHP-OVA coating ligand showed highest affinity, DDA-OVA the lowest, and the other haptens are almost equal. When comparing figure 1(A) and 1(B), it was not clear to elucidate any conclusion. The affinities were varied depending on the coating ligands which were derivatized to different structure and conjugated with different carrier proteins. The clear understanding is that shorter spacer arm showed lower affinity than longer one and the length of spacer arm plays an important role for steric hindrance for binding affinity between antibody and coating ligand. Same results were obtained in previous reports.¹⁷⁻¹⁹ Therefore, it is important to screen a best matching pair of antibody and coating ligand for affinity and displacement with DDT analytes.

The titer level of each antibody (PAb #1, PAb #2, PAb #3) was determined by competitive ELISA using five coating ligands (DDA-OVA, DDHP-OVA, DDCP-OVA, DDHH-OVA,

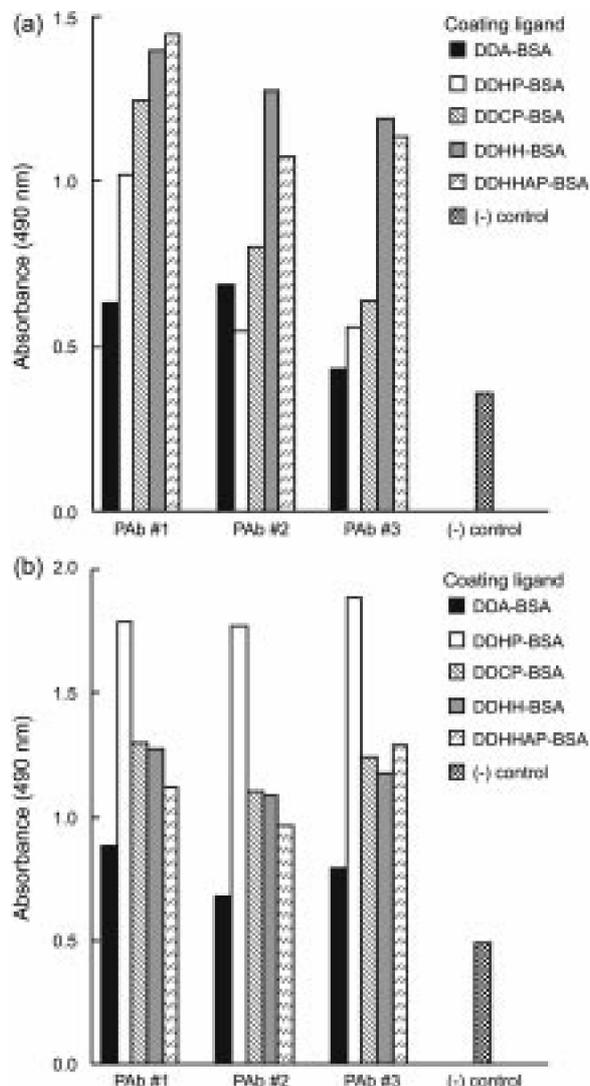


Figure 1. Comparison of binding capacities of DDT antibodies with various coating ligands. The PAb was used at 50 μL of 1/1000 diluted with PBS, and the coating ligand was used at 50 μL of 10 $\mu\text{g}/\text{mL}$. The binding capacities of DDT antibodies were compared using five kinds of BSA conjugated coating ligands (A) and five kinds of OVA conjugated coating ligands (B). PAb #1, #2 and #3 were obtained using immunogens of DDA-KLH, DDHP-KLH and DDCP-KLH, respectively.

Table 1. Chemical structures of DDT analytes and haptens prepared

	X	R
DDT	H	CCl_3
DDE	—	CCl_2
DDA	H	COOH
DDHP	OH	$(\text{CH}_2)_3\text{COOH}$
DDCP	Cl	$(\text{CH}_2)_3\text{COOH}$
DDHH	OH	$(\text{CH}_2)_4\text{COOH}$
DDHHAP	OH	$(\text{CH}_2)_4\text{CONHCH}_2\text{CH}_2\text{COOH}$

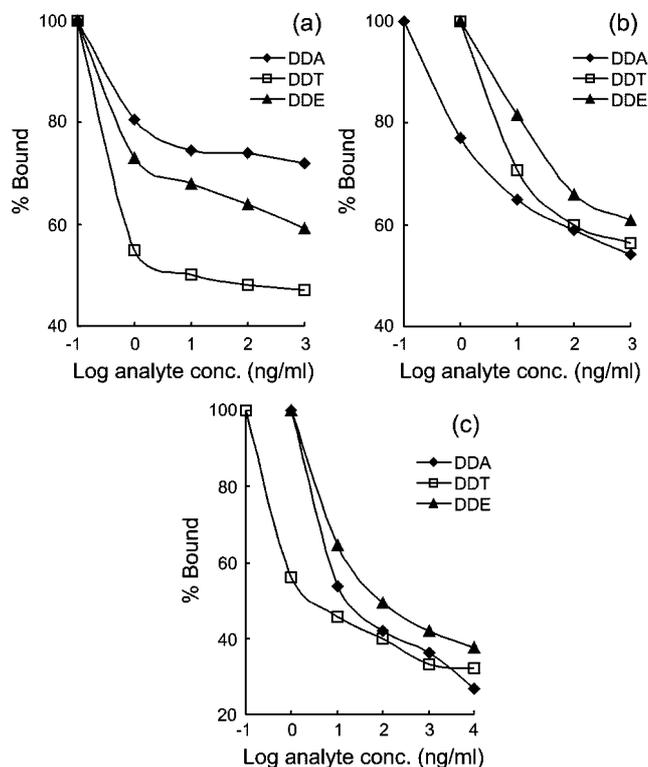


Figure 2. Dose-response curves of DDT analytes. The PAb (50 μL of titer level of each antibody in Table 2) and the coating ligand (50 μL of 10 $\mu\text{g}/\text{mL}$) were used for the dose-response curve of DDT analytes. PAb #1 (1:320 diluted) with DDCP-OVA coating ligand (A), PAb #1 (1:840 diluted) with DDHHAP-OVA coating ligand (B) and PAb #3 (1:1840 diluted) with DDHHAP-OVA coating ligand (C).

DDHHAP-OVA) to determine antibody concentration for the displacement reaction of DDT analytes (Figure 2). The antibody concentration that showed 70% of maximum binding response (absorbance at 490 nm) was determined as the

Table 2. Effect of coating ligand on the titration curves

Immunogen	Antibody	Coating ligand	Titer level ^a
DDA-KLH	PAb #1	DDA-OVA	190
		DDHP-OVA	540
		DDCP-OVA	320
		DDHH-OVA	320
		DDHHAP-OVA	840
DDHP-KLH	PAb #2	DDA-OVA	180
		DDHP-OVA	290
		DDCP-OVA	160
		DDHHAP-OVA	640
DDCP-KLH	PAb #3	DDA-OVA	80
		DDHP-OVA	5570
		DDCP-OVA	790
		DDHHAP-OVA	1680

^aThe titer level was determined by the value of antibody dilution fold on the 70% of maximum absorbance.

titer level (Table 2).

Competitive displacement curve was investigated to select a matching pair of antibody and coating ligand. Three matching pairs of antibody and coating ligand were screened and showed reactivities to DDT, DDA and DDE. Figure 2 showed the results for displacement responses of DDT analytes using a pair of antibody and coating ligand. They were PAb #1 with coating ligand of DDCP-OVA, PAb #1 with DDHHAP-OVA and PAb #3 with DDHHAP-OVA. BSA coating ligands cannot be obtained good competitive curves for DDT analytes. They seem to be strong affinity between antibody and coating ligand. The pair of antibody and coating ligand showed high binding response (Figure 1), but the bound complex of antibody and a coating ligand did not displaced by DDT analytes. DDHP-OVA coating ligand, which has the highest binding affinity to three antibodies (PAb #1, PAb #2, PAb #3) showed no displacement response which is similar to BSA-conjugated coating ligands. When antibody has a very strong affinity for the coating ligand, good sensitivity of an assay cannot be obtained because free analytes cannot be bound to the antibody easily in competition with the coating ligand. Colbert *et al.* also reported that increased sensitivity was achieved by reducing an antibody affinity of the tracer.²⁶ Therefore, OVA can be used as a good carrier protein for coating ligand, rather than BSA.

Table 3 showed the displacement reactivity of DDT analytes using selected pair of antibody and coating ligand. Detection limit of each analyte was obtained by the dose-response curve at the optimum condition of respective pair of antibody and coating ligand, and cross-reactivities of DDT analytes were compared by 70% inhibition concentration of DDT. Sensitivity was defined as the concentration of DDT analytes that yielded 70% of the maximum response from zero concentration on the dose-response curve. The cross reactivity values of DDT analytes with the pair of PAb #1 and DDCP-OVA were calculated as 100, 14, 12%, a pair of PAb #2 and DDHHAP-OVA were as 100, 156, 28% and

Table 3. Cross-reactivity of DDT analytes with a matching pair of antibody and coating ligand

Antibody	Coating ligand	Analytes	Cross-reactivity ^a (%)	Sensitivity ^b (ng/mL)
PAb #1	DDCP-OVA	<i>p,p'</i> -DDT	100	-
		<i>p,p'</i> -DDA	14	-
		<i>p,p'</i> -DDE	12	-
	DDHHAP-OVA	<i>p,p'</i> -DDT	100	2.8
		<i>p,p'</i> -DDA	156	0.4
		<i>p,p'</i> -DDE	28	4.3
PAb #3	DDHHAP-OVA	<i>p,p'</i> -DDT	100	0.3
		<i>p,p'</i> -DDA	11	3.0
		<i>p,p'</i> -DDE	7	3.4

^a% Cross-reactivity defined as % ratio of concentration for 70% displacement by DDT concentration 70 % displacement by analyte.
^bSensitivity defined as the concentration of DDT analytes that yielded 70% of the maximum response from zero concentration on the dose-response curve.

the pair of PAb #3 and DDHHAP-OVA were as 100, 11, 7% (Table 3). Therefore, PAb #1 and DDHHAP-OVA coating ligand exhibited high cross-reactivity values for the DDT analytes (DDT, DDA and DDE), but a pair of PAb #3 and DDHHAP-OVA exhibited most sensitive reactivity with DDT (0.3 ng/mL). The results indicate that the pair of PAb #1 and DDHHAP-OVA coating ligand was most responsible for DDA, and a pair of PAb #3 and DDHHAP-OVA was for DDT. Among 30 matching pairs of antibodies and coating ligands (a combination of three antibodies, five DDT derivatives, two carrier proteins), derivatives with longer spacer of DDHHAP and OVA conjugated coating ligand seem to be most applicable for the measurement of DDT analytes. Even though the sensitivity of dose-response was different in one order, above three pairs are responsible to determine the DDT analytes (DDT, DDA and DDE) simultaneously. This means that DDA-KLH that produced PAb #1 and DDCP-KLH produced PAb #3 were proper immunogens to produce DDT antibodies. These results suggest that a longer spacer for coupling of carrier protein is better to use as an immunogen for antibody production. Also, the DDHHAP-OVA coating ligand showed a better binding efficiency than the other coating ligands synthesized suggesting that the longer spacer in DDT derivatives produces good binding response as well as easily displaced by free competitor analytes. From these observation, we conclude that the length of spacer that is attached for coupling of carrier protein in immunogen and coating ligand could influence significantly the immunoreactivity in an indirect ELISA by affecting antibody production and dose-response immunoreaction. Therefore, careful investigations of hapten design and proper carrier protein were required to obtain the best dose-response immunoreaction.

In conclusion, DDA-KLH and DDCP-KLH immunogens were useful to produce DDT antibodies and DDHHAP-OVA was best coating ligand for the generation of dose-response curve. Using the selected pair of PAb #3 and DDHHAP-OVA coating ligand, DDT analytes can be detected simultaneously with the detection limit of 0.3 ng/mL for DDT and 3.4 ng/mL for DDA and DDE by an indirect enzyme immunoassay.

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