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Direct hapten coated immunoassay format for the detection of atrazine and 2,4-dichlorophenoxyacetic acid herbicides

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ABSTRACT

A novel immunoassay format employing direct coating of small molecular hapten on microtiter plates is reported for the detection of atrazine and 2,4-dichlorophenoxyacetic acid (2,4-D). In this assay, the polystyrene surface of microtiter plates was first treated with an acid to generate $-NO_2$ groups on the surface. Acid treated plates were further treated with 3-aminopropyltriethoxysilane (APTES) to functionalize the plate surface with amino groups for covalent linkage to small molecular hapten with carboxyl groups. The modified plates showed significantly high antibody binding in comparison to plates coated with hapten-carrier protein conjugates and presented excellent stability as a function of the buffer pH and reaction time. The developed assay employing direct hapten coated plates and using affinity purified atrazine and 2,4-D antibodies demonstrated very high sensitivity, IC_{50} values for atrazine and 2,4-D equal to 0.8 ng mL^{-1} and 7 ng mL^{-1} , respectively. The assay could detect atrazine and 2,4-D levels in standard water samples even at a very low concentration upto 0.02 and 0.7 ng mL^{-1} respectively in the optimum working range between 0.01 and 1000 ng mL^{-1} with good signal reproducibility (p values: 0.091 and 0.224 for atrazine and 2,4-D, respectively). The developed immunoassay format could be used as convenient quantitative tool for the sensitive screening of pesticides in samples.

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1. Introduction

Immunological methods such as enzyme linked immunosorbent assay (ELISA) are increasingly becoming important for pesticides residual analysis due to the high inherent selectivity of detecting molecules, i.e., antibodies. The fact that antibodies could be made virtually against any substance, and its usages in developing highly sensitive assay makes this approach quite useful for the analysis of these toxic molecules [1–4]. These assays apart from being highly specific, exhibit the desired sensitivity and accuracy for the detection of low molecular weight contaminants present in our environment. These assay formats are fast becoming a preferred choice to detect pesticide contamination in water because

they are specific, cost effective and rapid [5–8]. Development of sensitive immunoassay for pesticide detection depends primarily upon the quality of antibody used. For antibody production, hapten must be synthesized and coupled to carrier proteins. Both, conjugate preparation and immunization protocol greatly affect the quality of the generated antibodies. We have previously reported the synthesis of a well characterized hapten-protein conjugate by optimizing the hapten density (number of hapten molecules per molecule of carrier protein) to generate a highly specific antibody against atrazine [9].

In ELISA based applications, biological macromolecules are coated on a polystyrene surface by passive adsorption. The adsorption of these molecules to a polystyrene surface is due to intermolecular attraction forces (van der Waals forces,

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etc.), which are based on intramolecular electrical polarities. It has been observed that protein molecules immobilized on a hydrophobic polystyrene surface by passive adsorption lose their activity and suffer considerable denaturation [10]. These macromolecules are found to better retain their functional activity when immobilized through extended hydrophilic spacer arms, since sorption on the surface is substantially reduced [11]. A polystyrene surface can be modified to improve its hydrophilicity by incorporating various functional groups such as hydroxyl, amino, carbonyl, carboxyl, etc. on its surface [12–14]. In most hapten based ELISAs, hapten molecules are usually bound to polystyrene microtiter wells indirectly by coating the wells with protein–hapten conjugates, since direct attachment of hapten molecules to a polystyrene surface is not possible due to the lack of available functional groups on polystyrene. However, the formation of this conjugate is not always reproducible which makes it difficult to evaluate hapten–protein stoichiometry [15]. In order to avoid these drawbacks, a method for direct attachment of carboxylated hapten on polystyrene support for binding the biomolecules on modified polystyrene surface was earlier reported by our group [16]. In continuation with our earlier observation, we further describe a method for generating amino groups on polystyrene microtiter wells using simple one-step aqueous silanization method for binding carboxylated hapten to develop a highly sensitive immunoassay format for smaller molecules. This method allowed us to link carboxylated hapten to amine grafted polystyrene microtiter plates for the quantification of atrazine and 2,4-D pesticides. Highly sensitive hapten specific antibodies against atrazine and 2,4-D were generated and used in the present assay format showing high degree of assay sensitivity.

2. Experimental

2.1. Materials

2,4-Dichlorophenoxy acetic acid (2,4-D), atrazine and their analogues such as simazine, cyanazine, terbutryn, prometon, 2,4-dichlorophenoxybutyric acid (2,4-DB), 2,4-D methyl ester (2,4-DME), 2,4-D sodium monohydrate (2,4-DSMH), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), triclopyr, bovine serum albumin (BSA), *N,N'* dicyclohexylcarbodiimide (DCC), dimethylformamide (DMF), *N*-hydroxysuccinimide (NHS), 3-aminopropyltriethoxysilane (APTES), formaldehyde dioxane, keyhole limpet hemocyanin (KLH), Freund's complete adjuvant (CFA), Freund's incomplete adjuvant (IFA), horseradish peroxidase and goat anti-rabbit IgG-HRP conjugate were purchased from Sigma (India). 3,3',5,5' tetramethylbenzidine (TMB) was purchased from Bangalore Genei, India. Protein-A sepharose and sepharose-4B were procured from Pharmacia. All buffers were made in ultra pure Milli-Q water.

2.2. Hapten–protein conjugates preparation

Mercaptopropionic acid derivative of atrazine was synthesized and conjugated with carrier proteins (BSA and KLH) by the method as described earlier [7]. However, for conjugating 2,4-D with carrier proteins, the carboxylic acid groups of 2,4-D

were directly used to conjugate with carrier proteins using DCC as an activating reagent. For this, 10 mg 2,4-D together with 1.7 mg (15 μmol) NHS and 6.2 mg (30 μmol) DCC was dissolved in 1.3 mL DMF. The reaction mixture was incubated for 18 h at RT and then centrifuged at 12,000 RPM to remove the acyl urea precipitate. The activated hapten was then added dropwise to protein solution (1 mg mL⁻¹) made in 0.1 M borate buffer, pH 8 by selecting protein:hapten molar ratio equal to 1:40. The coupling reaction was carried out at 4 °C overnight under mild shaking and the conjugate was then dialyzed for 48 h against PBS. The extent of hapten conjugation to carrier proteins was determined by spectrophotometric technique [7] using Hitachi spectrophotometer.

2.3. Antibodies production and purification

The New Zealand white rabbits were immunized subcutaneously with 400 μg of hapten–protein conjugates emulsified in Freund's complete adjuvant for first immunization followed by booster doses prepared in Freund's incomplete adjuvant. The sera were collected, pooled and precipitated with 50% saturated ammonium sulfate under constant stirring at 4 °C followed by centrifugation at 10,000 RPM. The precipitate was dissolved in PBS and then dialyzed against PBS (pH 8.0) for 24 h at 4 °C. The solution was then passed through the protein-A sepharose column to isolate the IgG fraction of proteins. Bound antibody was eluted with 0.1 M glycine-HCl buffer (pH 2.5) and further purified by passing through BSA-sepharose column to remove anti-BSA antibodies from the fractions. The total recovery of eluted antibodies was determined by taking absorbance at 280 nm.

2.4. Antibodies reactivity on direct hapten coated and conjugated hapten coated microtiter plates

Microtiter plates were coated with hapten molecules directly as described in our earlier studies [16]. In brief, the plates were first acid treated with 47% (v/v) HNO₃ in concentrated H₂SO₄ for 30 min at room temperature to generate –NO₂ groups on polystyrene surface. The modified wells were then treated with 5% aqueous aminopropyltriethoxy silane (APTES) solution at pH 6.9 by first incubating at RT for 2 h and subsequently reacted at 62 °C to introduce amino groups on polystyrene surface. After APTES treatment, 250 nmol of activated 2,4-D and MPAD was added separately into each well of microtiter plates and incubated overnight at 4 °C. Blocking was done with 0.2 M formaldehyde by incubating the plates for 2 h at 37 °C to block the unbound sites of the PS surface. The reaction mechanism of direct coating of carboxylated hapten molecules on APTES treated PS surface is shown in Fig. 1. Microtiter plates were also coated with protein–hapten conjugates (2,4-D–BSA and MPAD–BSA) by adding 100 μL of each conjugate (5 $\mu\text{g mL}^{-1}$ in carbonate buffer) per well of microtiter plate and incubating the plates overnight at 4 °C. Non-specific sites on plates were blocked by treating the PS surface with 10% skim milk prepared in PBS for 2 h at 37 °C and washed three times with PBS (pH 7.4) for further reaction.

100 μL of specific antibodies (anti-2,4-D and anti-atrazine) was added into each well of the plates coated with direct hapten and also coated with hapten–protein conjugates. After

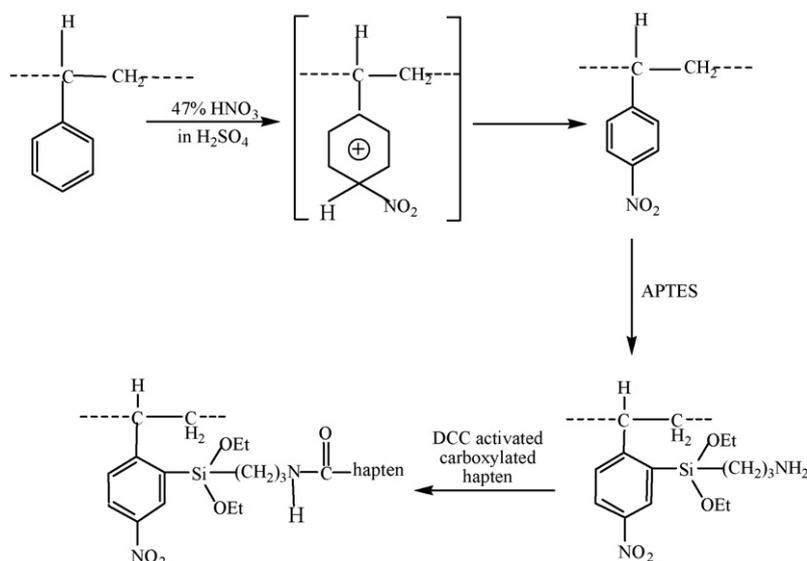


Fig. 1 – Reaction mechanism of direct coating of carboxylated hapten molecules on APTES treated PS surface.

2 h incubation at 37 °C, the plates were washed thoroughly with PBS and then 100 μ L of secondary antibody (goat anti-rabbit IgG-HRP) at 1:5000 dilution made in PBS containing 0.1% skim milk was added in each well. The plates were incubated for 1 h at 37 °C followed by the addition of TMB substrate for color development. Absorbance at 450 nm was measured using ELISA reader (Molecular Devices, USA).

2.5. Competitive inhibition using direct hapten and conjugated hapten coated microtiter plates

ELISA plates were coated with activated hapten (2,4-D and MPAD) and also with protein conjugated hapten (2,4-D-BSA and MPAD-BSA) as described above. The respective plates were then treated with specific antibody (0.1 μ g mL⁻¹) preincubated with different concentrations of respective free hapten (0.5 pg mL⁻¹ to 5 μ g mL⁻¹) for 2 h at 37 °C. After washing thoroughly, the plates were incubated with goat anti-rabbit IgG-HRP (1:5000 dilution) in PBS containing 0.1% skim milk for 1 h at 37 °C followed by the addition of TMB substrate (100 μ L well⁻¹) for color development. Absorbance was measured at 450 nm using ELISA reader (Microdevices, USA) and data analysis was performed by normalizing the absorbance using the following formula:

$$\% \frac{B}{B_0} = \frac{(A - A_{ex})}{(A_0 - A_{ex})} \times 100$$

where A: absorbance of hapten at standard concentration, A₀: absorbance at zero hapten concentration, and A_{ex}: absorbance at excess hapten concentration.

Cross-reactivity of related analogues were calculated on the basis of standard calibration curves in a range of ng mL⁻¹ to sub ng mL⁻¹ level. The data was normalized by %B/B₀ transformation and the specific hapten concentration yielding 50% inhibition was used to calculate the cross-reactivity according

to the formula:

$$\% \text{Cross reactivity} = \left(\frac{H}{C} \right) \times 100$$

where, H is the concentration of standard hapten at 50% B/B₀ and C is the concentration of cross reacting hapten/analogue at 50% B/B₀.

3. Results and discussion

3.1. Anti-hapten antibodies generation and purification

To develop a specific and sensitive immunoassay for the detection of pesticides, the crucial step is to generate good quality antibodies against target molecules. Hapten design and the choice of carrier protein are important in the process of generating specific antibodies against smaller hapten molecules such as pesticides, etc. These molecules are synthesized and conjugated with carrier proteins in such a way that they mimic the structure of the compound and contain a reactive group that can form a covalent linkage with the carrier proteins [9,17]. The method used in the present study utilized carbodiimide activation method for carboxylated haptens (MPAD and 2,4-D) to link to carrier proteins, which ensured stable cross-linking of haptens with proteins. Fig. 2 shows the reaction mechanism of conjugation of atrazine and 2,4-D with carrier protein (BSA) using carbodiimide method [7].

By selecting appropriate protein-hapten conjugates prepared at optimum molar ratio of protein:hapten (1:40), significantly high titers of anti-2,4-D and anti-MPAD antisera were observed (Fig. 3a and b). The titer of antisera was around 1 \times 10⁻⁶ and 2 \times 10⁻⁶ for BSA-2,4-D and BSA-MPAD immunized rabbits, respectively after 3rd booster dose of immunogens. Antibodies were first purified by protein-A sepharose followed by BSA-sepharose-4B column to remove anti-carrier (BSA) antibodies. The recovery of specific anti-

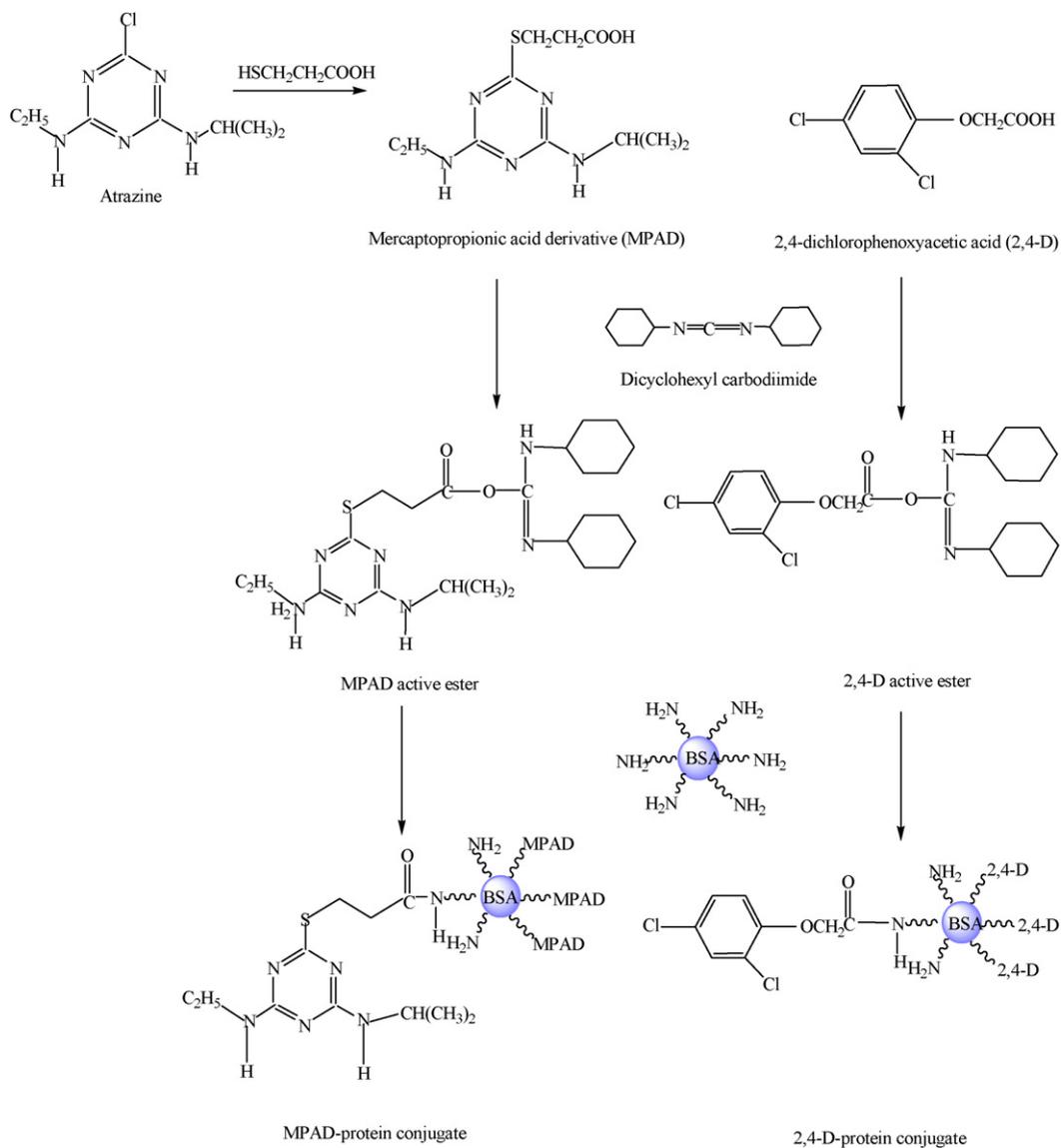


Fig. 2 – Schematic representation of activation of carboxyl group of 2,4-D and MPAD for coupling with carrier protein.

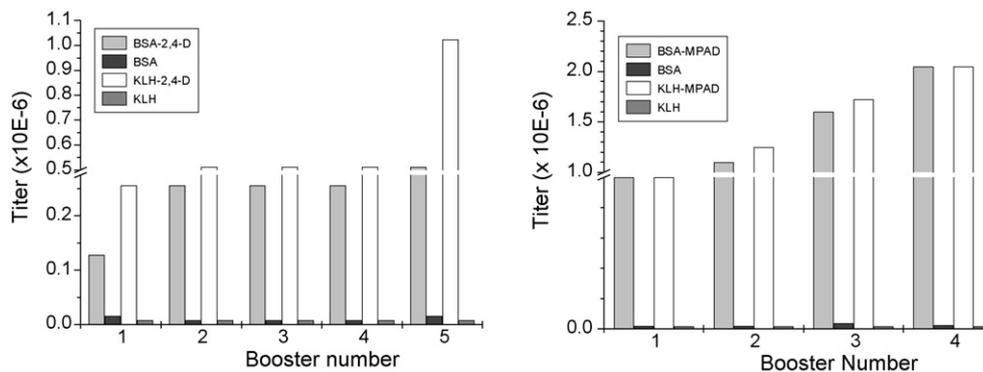


Fig. 3 – Antisera titer against (a) BSA-2,4-D and (b) BSA-MPAD. Titer value was calculated where the O.D. was 0.1 and 1.0 for BSA-2,4-D and BSA-MPAD, respectively.

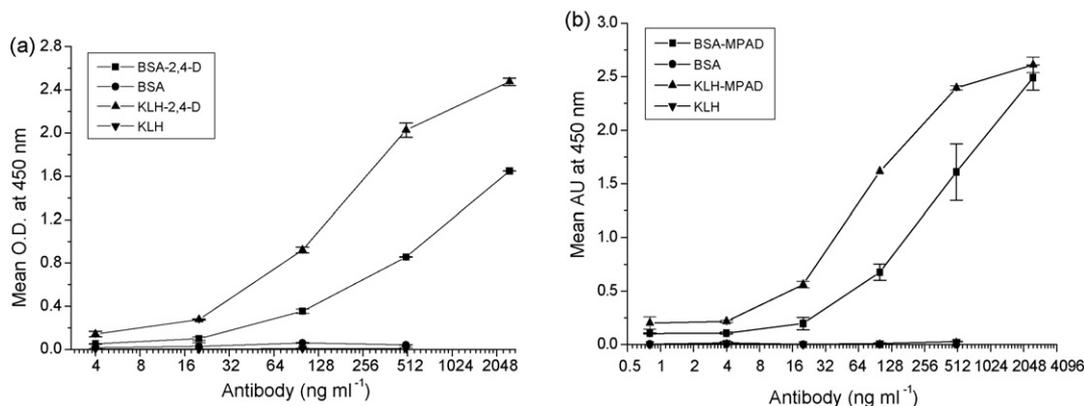


Fig. 4 – Dilution curves showing reactivity for BSA-sepharose-4B column purified anti-BSA-2,4-D antibody (a) and anti-BSA-MPAD antibodies (b) against (■) BSA-hapten, (●) BSA, (▲) KLH-hapten and (▼) KLH.

bodies (anti-2,4-D and anti-MPAD) after passing through sepharose-BSA column was approx. 8 mg mL⁻¹. Anti-2,4-D antibodies showed good reactivity against BSA-2,4-D and KLH-2,4-D conjugates. Similarly, anti-atrazine antibodies showed good reactivity for BSA-MPAD and KLH-MPAD. Both antibodies demonstrated a significantly low reactivity with carrier proteins (BSA and KLH) and showed good reactivity upto 6.25 ng mL⁻¹ and 0.8 ng mL⁻¹ for 2,4-D-BSA and MPAD-BSA conjugates, respectively (Fig. 4a and b).

3.2. Specificity and cross-reactivity of anti-hapten antibodies

Table 1 shows the sensitivity and cross-reactivity of anti-2,4-D antibodies with 2,4-D and its analogues, as determined by

using HRP-2,4-D and free hapten in competitive immunoassay format. Antibodies showed very high sensitivity and cross reactivity with 2,4-D (IC₅₀ equal to 0.7 ng mL⁻¹) in comparison to 2,4-DB (IC₅₀: 3.08 ng mL⁻¹), 2,4-DME (IC₅₀: 2.6 ng mL⁻¹) and 2,4-DSMH (IC₅₀: 2.0 ng mL⁻¹), respectively. The generated antibodies showed significantly low reactivity against 2,4,5-T (IC₅₀ 10.5 ng mL⁻¹) and triclopyr (IC₅₀ equal to 60 ng mL⁻¹). Similarly, anti-MPAD antibodies showed significantly high sensitivity and specificity against the target molecule, atrazine, and as well as for similar structured atrazine analogues such as prometon and simazine. The generated antibodies demonstrated lower cross-reactivity against cyanazine and terbutryn (Table 2). Comparison of the data from Table 1 and 2 shows that sensitivity and cross-reactivity patterns of both anti-2,4-D and anti-MPAD are different. The

Table 1 – Cross-reactivity of anti-2,4-D antibody with 2,4-D and its analogues

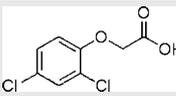
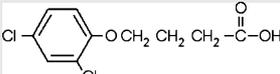
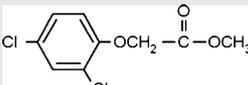
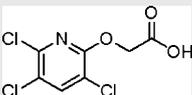
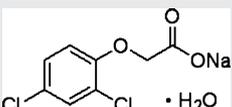
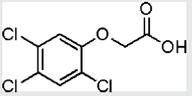
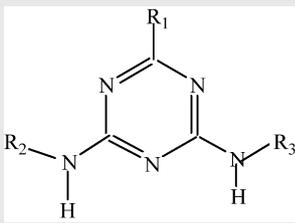
2,4-D and its analogues	IC ₅₀ value (ng mL ⁻¹)	Cross-reactivity (%)
2,4-D 	0.7	100
2,4-DB 	3.08	23
2,4-DME 	2.60	27
Triclopyr 	60.0	1
2,4-DSMH 	2.00	35
2,4,5-T 	10.5	7

Table 2 – Cross-reactivity of anti-MPAD antibody with atrazine and its analogues

Atrazine and its analogues		IC ₅₀ (ng mL ⁻¹)	CR (%)
			
MPAD	R1 = S(CH ₂) ₂ COOH, R2 = ethyl and R3 = isopropyl	0.10	100
Atrazine	R1 = chloro, R2 = ethyl, R3 = isopropyl	0.11	91
Prometon	R1 = methoxy, R2 and R3 = isopropyl	0.15	67
Cyanazine	R1 = chloro, R2 = ethyl, R3 = cyanopropyl	2.00	5
Terbutryn	R1 = thiomethyl, R2 = ethyl, R3 = tertbutyl	1.05	10
Simazine	R1 = chloro, R2 and R3 = ethyl	0.14	71

production of antibodies against small molecular haptens such as 2,4-D and atrazine requires preparation of hapten that mimics the structure of the analyte. The introduction of a linker in the hapten molecules to couple with carrier protein might increase the accessibility of hapten on the surface of the carrier protein for better recognition of these molecules by immune system. For the synthesis of MPAD for bioconjugation with protein, we incorporated a linker arm of three carbon atoms i.e., propionic acid derivative. However, in case of 2,4-D, the activated carboxyl groups of 2,4-D were directly used to link with carrier protein (Fig. 2). This may be one of the reasons for getting high titer of anti-atrazine antibodies with better sensitivity in comparison to anti-2,4-D antibodies.

3.3. Anti-hapten antibodies reactivity on direct hapten coated ELISA plate

Binding of purified polyclonal antibodies with target molecules (atrazine and 2,4-D) on hapten coated ELISA plates were checked by confirming the reactivity of antibodies at different concentrations between 1.6 μg mL⁻¹ and 1.56 ng mL⁻¹. Hapten coating on ELISA plate was first optimized by taking different hapten concentrations as described previously [16]. Fig. 5 shows the binding of anti-2,4-D and anti-MPAD antibodies on PS plates coated with direct hapten molecules (2,4-D and MPAD) at optimum hapten concentration 250 nmol well⁻¹. A linear increase in the signal intensity (OD values) was observed with different antibody concentrations on respective hapten coated plates. However, normal IgG (nonspecific antibody as a control) on hapten coated plate showed no signal intensity, thus establishing the sensitivity, reproducibility (*p* values: 0.091 and 0.224 for atrazine and 2,4-D respectively) and workability of newly developed direct hapten immunoassay format for pesticides detection.

3.4. Competitive inhibition assay for atrazine and 2,4-D

Competitive ELISA was performed using direct hapten coated and plates coated with hapten–protein conjugates using purified 2,4-D and atrazine antibodies at optimum antibody

concentration of 1.6 μg mL⁻¹ each. For this, each antibody was pre-incubated with different concentrations of target molecules, 2,4-D and MPAD and binding was checked with respective conjugated hapten coated plate and direct hapten coated plates. The results show that for hapten–protein conjugates coated plates, the IC₅₀ values (50% B/B₀) for 2,4-D and atrazine were observed approx. 70 ng mL⁻¹ and 12 ng mL⁻¹, respectively. However, the level of signal increased many folds upto 7 ng mL⁻¹ and 0.8 ng mL⁻¹ with the direct hapten coated plates for 2,4-D and atrazine respectively (Fig. 6a and b), and consequently the limit of detection upto 0.7 ng mL⁻¹ and 0.02 ng mL⁻¹ for respective target molecules. Thus, it was observed that the sensitivity of assay for pesticides detection in direct hapten coated format was significantly increased.

In immunoassay based pesticides detection, it is important to have the use of an antibody that demonstrates very high sensitivity as well as specificity. In many previous studies polyclonal antisera as such have been used for estimat-

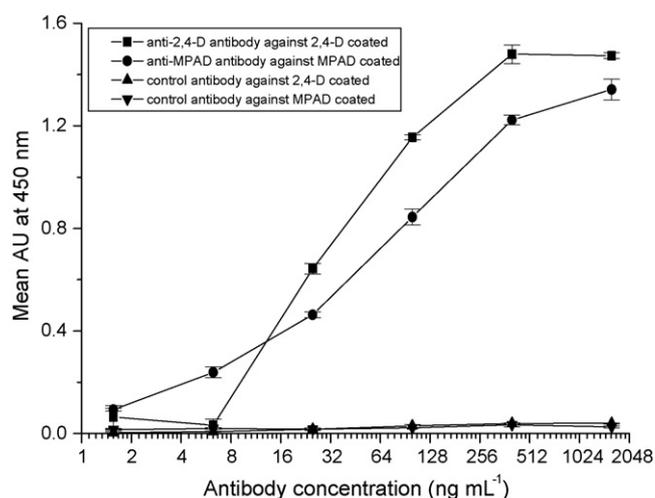


Fig. 5 – Binding of purified anti-MPAD and anti-2,4-D antibodies with target molecules (atrazine and 2,4-D) on hapten coated ELISA plates. The reactivity of antibodies was checked at different concentrations between 1.6 μg mL⁻¹ and 1.56 ng mL⁻¹.

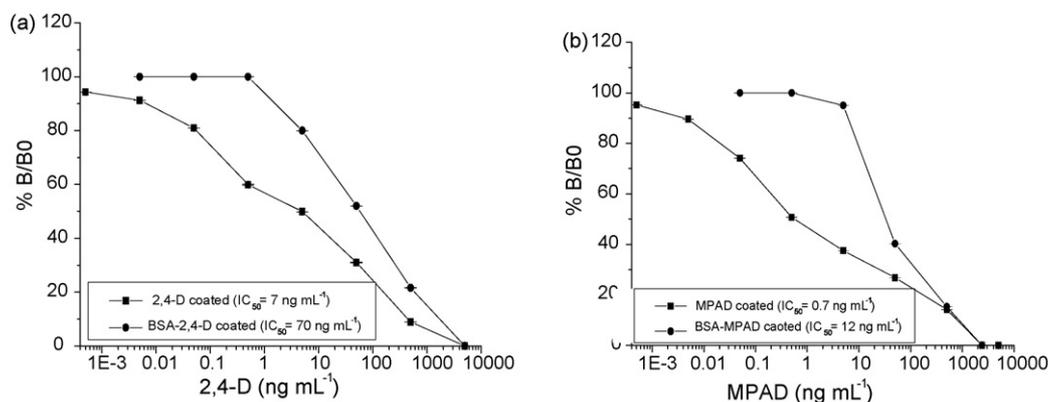


Fig. 6 – Competitive inhibition assay using conjugated hapten (BSA-hapten) and direct hapten coated plates for (a) 2,4-D and (b) atrazine. The curves show the dilution curve analysis for hapten concentrations between 0.5 pg mL^{-1} and $5 \text{ } \mu\text{g mL}^{-1}$. Free haptens were pre-incubated with specific antibody for 30 min before adding into microtiter plates.

ing the levels of different pesticides [18–23]. In the present study, we have demonstrated the use of highly purified antibodies using a combination of Protein-A sepharose column followed by passing through carrier protein column. This resulted in total recovery of about 75–80% specific anti-hapten antibodies. The relative affinity constant of antibodies, as calculated with the computer program [24] indicated that both anti-2,4-D and anti-MPAD antibody showed lower relative affinity using conjugate coated plates ($8.59 \times 10^7 \text{ L mol}^{-1}$ and $9.28 \times 10^8 \text{ L mol}^{-1}$) as compared to direct specific hapten coated plates ($1.80 \times 10^{10} \text{ L mol}^{-1}$ and $1.9 \times 10^{10} \text{ L mol}^{-1}$).

Competitive ELISA established the specificities of anti-2,4-D and anti-MPAD antibodies. Among all analogues of atrazine tested (Table 2), the cross-reactivity (% CR) of atrazine was observed very high approx. 90% in comparison to other analogues. The anti-MPAD antibody, as prepared by conjugating hapten through mercaptopropionic acid spacer, still recognized triazine compounds having chloro (Cl) moiety at R1 position (atrazine and simazine). This is in agreement to the similar observation, where it has been demonstrated that the sulfur of the spacer effectively mimics the Cl group [25]. The presence of two isopropyl groups at R2 and R3 position in prometon may contribute for the better reactivity with atrazine antibody unlike terbutryn, where only one terbutyl group was present at R3 position. In case of 2,4-D, the related analogues were different due to their ester, butyric acid and sodium monohydrate groups rather than the phenoxyacetic acid group, and hence in their cross-reactivity. The detection limit of our assay was thus observed much higher (0.260 ng mL^{-1} for 2,4-D) than the detection limit reported in the previous studies [26,27].

An enzyme-linked immunosorbent assay for small molecules, in general, needs conjugates of the hapten with large carrier protein for coating the wells of microtiter ELISA plates. The formation of such conjugates is not always reproducible. This makes it difficult to evaluate hapten–protein stoichiometry and to understand the precise orientation of the hapten on the protein [15]. Also, protein molecules while linked to hydrophobic polystyrene surface by passive adsorption might lose their activity and may suffer considerable

denaturation. These macromolecules are found to better retain their functional activity when immobilized through extended hydrophilic spacer arms, since sorption on the surface is substantially reduced. In an ELISA, the sensitivity of the assay depends to a great extent on the degree of antigen binding to the microtiter plates. The binding of hapten to the microtiter plates was examined using the direct hapten coated plates and by using hapten–protein conjugates on microtiter plates. The sensitivity of the assay obtained by using direct hapten coated plates was about 100 folds higher than the assay performed with hapten–protein conjugates with very high degree of reproducibility. This was mainly because of retention of functional activity of hapten molecules on polystyrene plates. No loss of functional activity of hapten molecules which is an organic moiety was observed, as reported in case of biomolecular immobilization on polystyrene plates.

We earlier described a method for generating amino groups on polystyrene microtiter wells using simple one-step aqueous silanization method [16]. The amine modified PS surface was used for demonstrating binding of carboxylated hapten for immunoassay applications. This method allowed us to link carboxylated hapten to amine grafted polystyrene microtiter plates for the quantification of atrazine and 2,4-D pesticides. Highly sensitive hapten specific antibodies against atrazine and 2,4-D were generated and used in the present assay format showing high degree of assay sensitivity.

Binding of hapten to microtiter plate was highest when wells were treated first with 47% HNO_3 in concentrated H_2SO_4 ($250 \text{ } \mu\text{L well}^{-1}$) followed by APTES treatment at pH 4 for making plates amino functionalized. This was confirmed by measuring the loading of antibody on hapten coated microtiter plates by ELISA. The results showed that the absorbance of antibody bound to hapten coated plate at pH 6.9 was 1.749 absorbance unit (AU) by using nitrated-PS wells in comparison to untreated PS surface which showed 0.63 AU at pH 6.9. The electron withdrawing nitro groups on PS surface drives the electrophilic attack of the silane group to its meta position enabling the binding of the APTES molecules to the PS surface, as demonstrated previously [16].

Another advantage of the direct hapten coated microtiter plate is its long term storage ability since the coated plate did not show any significant loss in its antigenicity with anti-hapten antibodies, unlike reported where the significant antigenicity of hapten was lost when glutamic acid coated plates prepared by the direct glutaraldehyde activation method were tested after 4 days storage [28].

4. Conclusions

In summary, we have developed a novel enzyme immunoassay format in which small molecular haptens with carboxylic groups were coated directly on microtiter plates for the detection of pesticides. Well characterized antibodies against atrazine and 2,4-D were generated by selecting appropriate hapten–protein conjugates and were used in the present assay format to develop a highly sensitive ELISA for pesticide detection. The developed assay format was highly sensitive and could detect atrazine and 2,4-D levels in standard water samples at concentrations as low as upto 0.02 and 0.7 ng mL⁻¹, respectively with good signal precision. The systematic approach described in this paper for antibody generation, characterization and assay optimization, particularly exploiting hapten-coated plate may find wide application as a convenient quantitative tool for sensitive screening of pesticides in contaminated water or soil samples.

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