SUMMARY OF THE THESIS

Phenoxy acid herbicides have been extensively used for the control of weeds in agriculture. Phenoxy acid herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyacetic acid (MCPA), dichlorprop (DCPP), and mecoprop (MCPP) are among the 10 most important pesticides. Due to their high solubility in water, phenoxy acid herbicides easily enter surface or ground waters through natural drainage or infiltration, and have been widely detected in groundwater. Because of the toxicity of phenoxy acid herbicides and their metabolic products, monitoring their concentration in water is important. Due to their widespread use and potential health hazards, it is important to monitor their presence, even at very low levels (ng/ml level) in the environment and foods. The most commonly applied methods for the determination of these compounds are gas chromatography (GC) after derivatization or liquid chromatography (LC), where a preconcentration step is usually needed. Various procedures have been developed for the extraction and preconcentration of phenoxy acid herbicides from water samples.

Among currently available analytical methods, the immunoassay technique is the most easily adaptable for analysis of large sample loads and this is indeed a sought-after criterion. Immunochemical methods are popular in pesticide residue analysis because they are rapid, sensitive, specific, cost-effective and field applicable. Immunoassay methods have been used to detect a number of pesticides, such as organochlorines, organophosphates, triazines, phenoxy acids, and others, in water, soil, and foods.

The present study was aimed at developing a sensitive immunochemical technique for the detection of pesticide in aqueous media. The first and foremost requirement for developing an immunobiosensor is generation of antibodies, which bind to the target analyte with high degree of specificity. As pesticide molecules are small in size to be able generate antibodies against then, therefore these molecules need to be conjugated to large protein molecules to be able to act as immunogen.

Immunization with this conjugate results in production of antibodies specific to the whole conjugate i.e., against immunogenic carrier protein and the hapten (small molecule) linked to the conjugate. The specificity and selectivity of the antibodies produced in response to immunization with a protein-hapten conjugate depends primarily upon the structure of the small hapten attached to the conjugate molecule. To achieve the defined objectives major emphasis was given to
a) Synthesis and characterization of bioconjugates for generation of specific and sensitive antibodies.
b) Characterization of antibodies in terms of specificity for use in immunoassay development.
5.1 Preparation and characterization of immunogens

For generation of specific and sensitive antibodies against small molecules such as drugs, pesticides, etc., requires the small molecule (hapten to be conjugated to a large protein molecule. The method used in this study utilized the carbodiimide method for carboxy acid haptens to link to the free amino groups of BSA. This approach ensured stable cross-linking of haptens with protein along with N-acylurea formation. The hapten-protein conjugates of different molar ratios (D1-D5 for 2,4-D-BSA and B1-B5 for 2,4-DB-BSA) were prepared. Fluorescence emission spectra for each hapten-protein conjugate (D1-D5 and B1-B5) in the range of 300–400 nm after excitation at 290 nm (excitation of tryptophan residues only) was recorded. Conjugates with increasing molar ratios of hapten-protein presented a linear decrease in fluorescence intensity. Protein molecules excited at 290 nm emit a fluorescence signal at 340 nm, primarily because of the number of tryptophan residues present in the protein. The decrease in fluorescence intensity at this wavelength is due to quenching of tryptophan intensity with increase in hapten density, i.e., increase in the number of amide bonds formed between the surface lysine groups of the protein and carboxylated hapten. The gradual decrease in fluorescence intensity with increasing hapten density was mainly due to change in the environment of tryptophan residues causing quenching of tryptophan intensity. This gradual shift in the fluorescence signal because of quenching of tryptophan intensity with different conjugates thus confirms the course of hapten-protein conjugation. These results were further validated by native-PAGE and MALDI-TOF MS. In the native-PAGE, a gradual shift in the protein bands with respect to native protein (control) was observed with conjugates of higher hapten density. Also, the number of hapten molecules linked to protein was further confirmed by analyzing the mass spectrum obtained with the MALDI-TOF spectrometer. Conjugation density for each hapten in increasing molar ratio resulted in a detectable increase in the MW of the conjugate as determined by observing the peak shift of mass spectra for hapten-protein conjugates. The MW of each conjugate was calculated from the centroid of the peaks. BSA molecule with MW of 66 531 Da showed a gradual shift in mass peaks with conjugates made with different molar ratios of haptens. The incremental change in MW due to incorporation of hapten molecules to protein corresponds to the number of hapten molecules/protein molecule. The effect promoted by hapten-protein conjugation density as determined by MALDI-TOF upon the fluorescence intensity of the intrinsic tryptophan chromophore molecules was plotted for protein conjugates 2,4-D-BSA and 2,4-DB-BSA. The correlation between protein conjugation densities as determined by MALDI-TOF for different ratio of hapten-protein conjugates and the relative fluorescence intensity for
conjugates shows the linear relationship between hapten density and relative fluorescence intensity. A standard protein-hapten conjugate made by reacting hapten and protein in a ratio 1:30 was fitted in the standard curve, and corresponding fluorescence intensity was measured. By correlating with the relative fluorescence intensity from the standard curve, the hapten density of the conjugate was determined.

The hapten density of the conjugate is an important parameter that generally defines the quality and quantity of antibody produced. It has been observed that higher antibody titer with moderate antibody affinities is obtained with hapten density approximately in the range of 20 molecules(carrier protein) (for D4 conjugate). It has been reported previously that lower hapten density in a conjugate induces a slower immune response, while higher substitution (more than 30 hapten/protein molecules) generally resulted in an IgM response and produced antibodies of lower affinity. By taking well-characterized hapten-protein conjugates, prepared at a protein: hapten molar ratio of 1:40, significantly high titer of anti-2,4-D and anti-2,4-DB antibodies were obtained (around 2 X 10^{-5} to 1 X 10^{-6} from the third booster onward). Antibodies were purified by protein-A sepharose, and then using a BSA-sepharose column to remove anti-carrier (BSA) antibodies. Antibody recovery with a protein-A sepharose-4B column was around 10-12 mg/ml. Upon further purification using a BSA-sepharose column, the recovery was approximately 8 mg/ml of specific antibodies. In immunoassay based pesticide detection, it is important to use an antibody that demonstrates very high sensitivity as well as specificity. In many previous studies, polyclonal antisera, as such, have been used to estimate the levels of different pesticides.

5.2 Preparation and Characterization of Immunoreagents

Immunoreagents were prepared for developing four types of immunoassay mainly, using enzyme/fluorophore/Gold nanoparticle as labels for fluorescence/chemiluminiscence/CNT/dipstick based immunoassay formats.

HRP/FITC molecule was conjugated to the hapten molecules and their stability, fluorescence yield was checked to avoid quenching of fluorescence. Carbohydrate moieties of HRP were a suitable site for binding of haptens through linker arms, without losing the specific activity of the enzyme.

Colloidal gold particles (15-20 nm) were found suitable for development of immunoassay because of their unique size dependent electronic and optical properties. Use of smaller particles lead to loss of sensitivity shift in the plasmon resonance intensity. Similarly, larger particles did not give good colour because of aggregation problem. It was seen that the optimum pH and optimum amount of protein were essential for formation of
good conjugates. It was also seen that the number of colloidal gold particles stabilized by proteins, having greater number of free sulfide groups, was larger as compared to normal proteins. The suitability of these immunoreagents was determined by their efficient binding with different antibodies.

For Chemiluminiscence based assay, different sizes of colloidal gold were used to test the CL intensity viz. 16 nm, 30 nm, and 60 nm. It was shown that the CL intensity of 30 nm GNPs showed the highest reading as compared to that of 16 and 60 nm particles. It was attributed that the size variation in gold nanoparticles effect their catalytic behavior since at larger size there is decrease in active surface area of nanoparticles leading to decrease in the catalytic efficiency of GNPs.

5.3 Antibody Production and Characterization

a) Antiserum characterization

Antibody against BSA-2,4-D and BSA-2,4-DB were generated in rabbit and in chicken. The antisera produced was specific to 2,4-D as it was reacting very little to OVA with respect to OVA-2,4-D and the concentration of 2,4-D antisera increased gradually from the first booster to the third booster dose. Incase of BSA-2,4-DB antisera also same results was obtained, with the 2,4-DB antisera reacting to OVA-2,4-DB. Antibody generated in chicken was also checked for reactivity to the target molecule and found to be specific.

c) Antibody purification

Purification of antibodies from rabbits was done using protein A column. Antibodies extracted from eggs contain many different types of protein such as α- and β-lipovitellines (70%), phosvitine (16%) and low-density lipoproteins (12%).

We checked the purity of the IgY antibody eluted through T-gel chromatography by SDS-PAGE gel electrophoresis. Only two bands were observed one at 70 KDa heavy chain and another at 25 KDa light chain that means all the other proteins present were removed after passing through T-gel. IgY antibodies have a molecular weight of 180 KDa as against 150 KDa in IgG. Dot blot also confirmed its specificity towards 2,4-D.

5.4 Immunoassay Development

a) ELISA

Direct immunoassay was performed using Rabbit anti-2,4-D antibodies as immobilized capture antibodies. The sensitivity and cross-reactivity of anti-2,4-D antibodies with 2,4-D and its analogues, was 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 (IC$_{50}$ equal to 0.7 ng/ml) in comparison to 2,4-DB (IC$_{50}$: 3.08 ng/ml), 2,4-DME (IC$_{50}$: 2.6 ng/ml) and 2,4-DSMH (IC$_{50}$: 2.0 ng/ml), respectively. The generated antibodies showed significantly low reactivity against 2,4,5-T (IC$_{50}$ 10.5 ng/mL) and triclopyr (IC$_{50}$ equal to 60 ng/ml).

Indirect ELISA was performed whereby antibody was pre-incubated with different concentrations of target molecules. 50% B/B$_0$ signal (IC$_{50}$ value) was observed at 30 ng/ml of 2,4-D concentration for IgG 2,4-D. While IgG 2,4-DB showed an IC$_{50}$ value of 7 ng/ml for 2,4-DB.

It was demonstrated that anti-2,4-D antibody showed good reactivity (IC$_{50}$) with 2,4-D (30 ng/mL), 2,4-DB (31 ng/mL) and 2,4-DB-ME (2,4-DB methyl ester) (31 ng/mL) respectively. Similarly, anti-2,4-DB antibody showed very good reactivity with 2,4-DB and 2,4-DB-ME (IC$_{50}$ values: 7 ng/mL and 32 ng/mL respectively). For 2,4-D SM (2,4-D sodium monohydrate) and 2,4-D, the reactivity was very low. The change in IC$_{50}$ values for related analogues with anti-2,4-D and anti-2,4-D antibodies were attributed due to their ester, butyric acid or sodium monohydrate groups linked to major ring.

d) Dipstick assay

The lateral flow Dipstick assay was useful for rapid assay for screening of samples under field conditions. Without the use of any sophisticated instruments a visually detectable limit between 25-50 ppb was achieved. Other groups have used enzyme labeled tracers for increasing the sensitive of the assays but the main disadvantage is the multiple steps involved in the process and also limits the field applicability part.

c) Direct Plate coat ELISA

Formation of protein hapten conjugates is not always reproducible and in performing ELISA with these conjugates getting reproducible results is very difficult. To minimize the error, hapten was directly bound to the microtiter plates and compared with by using hapten–protein conjugates on microtiter plates. The sensitivity of the assay using direct hapten coated plates was about 20 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. The detection limit of our assay was thus observed much higher (0.7 ng/ml for 2,4-D) than the detection limit reported in the previous studies.

d) Chemiluminiscent assay

Chemiluminescence (CL) method has been applied for the determination of organophosphorus pesticides residues during recent years due to its high sensitivity, rapid
assay speed and simple instrumentation. Influence of the size of the colloidal gold on chemiluminiscent intensity was measured for three sizes of particles viz. 16 nm, 30 nm, and 60 nm. It was shown that the CL intensity of 30 nm GNPs showed the highest reading as compared to that of 16 and 60 nm particles. It was attributed that the size variation in gold nanoparticles effect their catalytic behavior since at larger size there is decrease in active surface area of nanoparticles leading to decrease in the catalytic efficiency of GNPs.

Competitive inhibition using IgG 2,4-D-CG on microplate based inhibition assay was developed. Different concentrations of 2,4-D were prepared in the range of 0 to 2000ng/mL for the inhibition assay. The limit of detection was calculated to be around 3 ng/mL with IC\textsubscript{50} value of anti-2,4-D antibody as determined by standard ELISA protocol.

e) Fluorescence based assay

Inhibition assay using different concentration of 2,4-D with FITC conjugated anti 2,4-D antibody showed IC\textsubscript{50} was found to be at 3.6 ppb and limit of detection was at 1.3 ppb.

Fluorescence polarization based inhibition assay in ELISA plate using IgY, showed an IC\textsubscript{50} at 13 ng/ml. Reactivity and optimum dilution of the anti-2,4-D-BSA IgY antibody against 2,4-D-EDF molecule was checked. It showed a K\textsubscript{d} value of 6.169X10\textsuperscript{-5} M.

Inhibition assay showed IC 50% for 2,4-D was 78 ng/ml in borate buffer and with 2,4-D spiked in tape water it showed IC 50% at around 90 ng/ml. It was demonstrated that anti-2,4-D antibody showed IC\textsubscript{50} value for 2,4-DB (130 ng/ml), 2,4-DB Methyl (600 ng/ml), 2,4,5 Trichlorophenoxyacetic acid (800 ng/ml) and that with Atrazine at 1000 ng/ml respectively.

f) Liquid-gated field effect transistor (LGFET)

We have developed a LGFET microfluidic based biosensor and methodologies, direct, indirect and competitive immunoassays for the detection of a commonly used herbicide 2,4-D. The detection as extracted from the drain current response of the LGFETs yielded a real-time, label-free detection of free 2,4-D molecules with extremely low detection limits of \approx 500 fM (0.5 part-per-trillion) and 50 pM (50 part-per trillion) in soil extract and buffer solution, respectively. These detection limits are much lower than the maximum residual limit of 500 nM (500 part-per-billion) for the 2,4-D established by the WHO in drinking water.

5.5 Conclusions

2,4-D and 2,4-DB was conjugated to protein molecule for antibody generation and assay development. Estimation of hapten-protein conjugation efficiency was essential for determination of numbers of hapten on the protein molecule. A new fluorescence-based
method was described to determine the extent of haptenization in a hapten-protein conjugate. The method is very simple and fast, and does not require any sample preparation. The protein conjugates were further characterized by other established methods, such as MALDI-TOF MS, electrophoresis, and spectrophotometry. These well-characterized hapten-protein conjugates were used as immunogens for specific antibody generation. The generated antibodies showed very good reactivity with target molecules and were used in immunoassay format. A detection limit of 4 and 3 ng/ml was demonstrated for 2,4-D and 2,4-DB using anti-2,4-D and anti-2,4-DB antibodies, respectively. The antibody could be successfully used for large-scale screening of chlorophenoxyacetic acid pesticides in samples.

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. The developed assay format was 100 times more sensitive than conventional assay and could detect 2,4-D in standard water samples at concentrations as low as up to 0.7 ng/ml, with good signal precision.

We describe an immunoassay format using the catalytic properties of gold nanoparticles in the luminol-silver nitrate-gold nanoparticle based chemiluminescence system. Citrate reduced gold nanoparticles (30 nm) were used to label specific anti-2,4-D antibody produced in our lab to make immunonanoprobes. Parameters such as pH and type of buffer used were optimized for enhanced CL intensity. Competitive inhibition assay showed the level of detection of 2,4-D in standard water samples around 3 ng/ml. Compared with the conventional ELISA based methods, CL immunoassay methods based on gold nanoparticles, is simple, sensitive, and uses relatively inexpensive reagents.

The use of IgY antibodies is important since this class of antibody shows higher thermal stability and negligible reactivity with mammalian cross-reactants. Due to this property of IgY antibody, on field applicable devices are possible. High affinity and specificity IgY was developed and demonstrated that this Ab could be useful for FPIA and another immunoassays with high sensitivity and specificity. Because of its simplicity and speed, the developed FPIA could monitor target molecules in less than 10 min, which make it ideally suited for on-site field screening of these pesticides with handheld fluorescence polarizer.

Dipstick assay was developed for visual detection with unaided eye which are simple and fast to use. Here we developed an all-plastic biosensor comprising laminated single-walled carbon nanotubes as the active element and its conductance modulation in a liquid-gated field effect transistor, as the principle of transduction, for the detection of 2,4-
olorophenoxy acetic acid (2,4-D) herbicide. The reported biosensor is capable of forming real-time label-free detection of analytes in liquid environment. This biosensor which relies on immunoassay principle for specificity is able to detect down to 500 fM levels in soil samples.

Our results show that the choice between a suitable method for detection and monitoring depends upon the need of the individual. If a large number of samples are to be analyzed with low sensitivity one can use dipstick or ELISA is the method of choice for sensitivities. In ELISA assays around 24 samples in triplicate can be run simultaneously along with standard samples. The immunosensors are superior in terms of sensitivity and running time when small number of samples are to be analyzed with sensitivity.