Chapter 6

Summary and Conclusion
6.1 IMMUNOBIOSENSOR DEVELOPMENT AND CHARACTERIZATION

We aimed at developing an immunobiosensor for the detection of pesticides in aqueous media. Parathion and Atrazine being two of the most widely used pesticides all over the world was chosen as a target molecule. The first and foremost requirement for developing an immunobiosensor is generation of antibodies, which bind to the target analyte with high degree of specificity. The antibody generation comprised of two main parts,
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.

The tricky part is preparation of a suitable antigen for immunization of warm-blooded animals that leads to generation of specific antibodies. The complexity arises from the fact that small molecules (<1000D) are unable to initiate an immune response. Thus the small molecules (hapten) need to be conjugated to an antigenic carrier protein. 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.

6.1.1. HAPTHEN DESIGN AND SYNTHESIS

Parathion, with a molecular weight of 291.3, and atrazine, with a molecular weight of 215.7, were conjugated to a well known antigenic carrier protein BSA. The preparation of carrier protein-hapten conjugate requires that there are reactive functional groups on both the protein and Hapten molecule. Parathion does not have any functional groups that can be used for conjugation with carrier protein. So amino groups (-NH₂) and Carboxyl (-COOH) were chemically introduced into Parathion molecule without changing the major salient features of the molecular structure. Similarly, at least one of the groups attached to the central ring in Atrazine was removed to provide a site for attachment of a linker arm having a reactive functional group at the other end.
6.1.2. **Preparation and Characterization of Immunogens**

BSA-hapten conjugates were prepared using Amino Parathion with a molar excess ratio of 1:40 (protein: hapten), for immunization of animals and coating antigens for characterization of sera and antibodies. Use of a two step, carbodiimide active ester activation of carboxyl groups using DCC and NHS, conjugation procedure gave us flexibility to react proteins with activated hapten solution easily.

The hapten density on the BSA-AP and BSA-MPAD conjugates was determined by physical mass spectrometry using MALDI-TOF method. It was observed that the SDS-PAGE method was not suitable for determination of hapten densities because of its low sensitivity. It was observed that only conjugates having at least >5% change in mass gave detectable shift in SDS-PAGE band position. So the SDS-PAGE method was not able to detect hapten densities as low as those detectable by MALDI-TOF methods used.

We observed a decrease in the intrinsic protein fluorescence with increase in hapten density. The quenching of fluorescence was correlated with hapten density and the resulting mathematical equation from the density Vs relative fluorescence intensity gave good approximation of hapten density on conjugates prepared with intermediate protein: hapten molar reaction ratios. So we propose here a simple fluorescence based method for determination of Hapten densities.

We also observed a blue shift in the emission maxima of the BSA-MPAD conjugates with increase in the hapten density. This indicated a movement of tryptophan molecules towards a more hydrophobic region and thus indicating a change in the conformation of carrier protein brought about by conjugation with the hapten molecules.

The change in the structure of the carrier molecule on conjugation with the hapten is directly responsible for the nature of immune response, since binding of the hapten molecules with the protein carrier molecule either disrupts or appends the normal antigenic determinants of the carrier replacing them with haptenic determinants which are responsible for formation of hapten specific antibodies. Hence structural characterization of the carrier protein-hapten conjugate can help one choose the severity with which one wants to target the immune system to produce hapten specific antibodies.
6.1.3. Preparation and Characterization of Immunoreagents

Immunoreagents were prepared bearing Enzyme Linked Immunosorbent Assay (ELISA) in mind. This type relies on the enzymatic activity. It was confirmed that the carbohydrate moieties of HRP were a suitable site for binding of haptens through linker arms, without losing the specific activity of the enzyme.

6.1.4. Antibody Production and Characterization

The immunogens BSA-AP and BSA-MPAD, separately, were used to immunize rabbits. The antibody collected was checked for cross reactivity with different pesticides and it was observed that the anti-BSA-AP and anti-BSA-MPAD antibody apart from recognizing Parathion and atrazine, respectively, showed cross reactivity with the pesticide.

6.1.4.1. Antibody purification

The purification of Antibody was done in three steps:

In the first step precipitation of Antibody fraction and removal of unwanted serum proteins was carried out using Saturated Ammonium Sulfate precipitation method.

In the second step Protein-A affinity purification was carried out to purify total Antibody content from the serum. Antibody extracted by SAS precipitation method was used for loading on the protein-A affinity column. The concentration of the purified antibodies was determined using OD$_{280}$.

In the third step, hapten specific antibodies were purified using BSA column, at the ratio of 4 mg to 1 ml of matrix bed volume to obtain specific antibodies. Specific antibodies were eluted using different elution buffers, passing through the column at a flow rate of 1 ml/min. The eluted antibodies were dialyzed and its concentration was measured spectrophotometrically by taking OD at 280nm. However it was observed that the overall affinity of the hapten specific antibody was lower than the total Antibody purified from the serum. This may be because the greater avidity of the different antibody populations in the polyclonal antibody pool also contributes towards the determination of overall affinity of the antibodies while in case of hapten specific antibodies only single type of antibodies are available and hence a reduced affinity constant.

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6.2. IMMUNOASSAY DEVELOPMENT

ELISA: A highly sensitive ELISA was developed for detection of Pesticides. Using both Direct and Indirect formats, it was observed that the best sensitivity was obtained when Rabbit anti-AP antibody was used against conjugates prepared using BSA derivative of Parathion. Similar results were obtained for atrazine.

6.3. FUTURE PERSPECTIVES

With an increase in public awareness about the hazards posed by the toxic chemicals dumped daily in the environment, development of fast and cost effective methods for the environmental monitoring is the need of the hour. Immunobiosensors are proving to be an efficient way of monitoring environment not only for large pathogens but also for small molecules such as drugs, toxins and pesticides etc.

The essence of Immunobiosensors lies in the fact that antibodies can be produced against any given target molecule provided it can initiate an immune response on its own or can be made to initiate an immune response in warm-blooded animals. The generation of specific antibodies is the prime hurdle in the Immunobiosensors development and once the antibodies are formed, they can be used in a variety of devices for environmental monitoring.

The one area where development of the Immunobiosensors is lacking is fast and efficient production of antibodies having reproducible activity. The problem can be countered using molecular biology techniques where production of antigen binding fragments of antibodies specific to a given target is possible. It is, at least theoretically, possible to harvest the genetic information of an animal to produce target specific antigen binding antibody fragments but the time and effort required are tremendous taking into account the screening and optimization of conditions for functional protein production system.

Another area where development if possible is the multianalysis, where many targets can be simultaneously analyzed using the FIA micro fluidic biosensors, microarrays and Capillary electrophoresis are some promising techniques that can be coupled with the selectivity and sensitivity of the antibodies for making such a multianalysis
biosensor a reality. This brings us to another area of development of biosensors where miniaturization of the systems is the key to develop fast, cheap and reliable biosensors for monitoring.