The study presented in this dissertation deals with the development of biochemical techniques for ultra-sensitive detection of Sulfonylurea herbicides (SUs) using nanoparticles. The first and foremost requirement for developing an biochemical technique for detection of pesticides is the generation of specific receptor molecules (antibodies and/or aptamers), which can bind to the target analyte with high degree of specificity. The nanoparticles were synthesized using different chemistries by both biological and synthetic route. These receptors were used for developing nanoparticles mediated ultra-sensitive bio sensing platforms for SUs detection.

Sulfonylurea herbicides have been extensively used for the control of weeds in agriculture. Sulfonylurea herbicides such as bensulfuron are among the 10 most important pesticides. Due to their high solubility in water, sulfonylurea herbicides easily enter surface or ground waters through natural drainage or infiltration, and have been widely detected in groundwater. Because of the recent reports of extensive yield loss in non-target crops of sulfonylurea herbicides and their metabolic products, monitoring their concentration in water and ground water is of importance. The most commonly applied methods for the determination of these compounds are gas chromatography (GC) after derivatization or liquid chromatography (LC), where a pre-concentration step is usually needed. Various procedures have been developed for the extraction and pre-concentration of sulfonylurea herbicides from water samples.

Among currently available analytical methods, the immunoassay technique is the most easily adaptable to the analysis of large sample loads and this is indeed a sought-after criterion. Immunochemical methods are increasingly popular in pesticide residue analysis because they are rapid, sensitive, specific, and cost-effective. 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 biosensor for the detection of herbicide in aqueous media. The first and foremost requirement for developing an biosensor is generation of receptor molecule (antibody or aptamer), 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. For successful immunoassay development, main emphasis should be 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.

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.

5.1 Synthesis and characterization of nanoparticles

Nanoparticles were synthesized using both biological and chemical route. The process of synthesis of well-dispersed nanoparticles using a highly efficient microorganism *Stenotrophomonas maltophilia* was reported for the 1st time leading to the development of an easy bioprocess for synthesis of GNPs of desired size and shape. The results presented

demonstrate that a specific NADPH-dependent enzyme present in the isolated strain reduces Au^{3+} to Au^{0} through an electron shuttling mechanism leading to the synthesis of nearly monodisperse GNPs. This green route of biosynthesis of GNPs is a simple, economically viable and an eco-friendly process.

Chemical synthesis of gold nanoparticles of different sizes was carried out using different chemistries for ther subsequent bioconjugation for receptor generation as well as bioassay development.

Gold nanoparticles coated with palladium dots (Pd@Au) bimetallic nanostructure were synthesized and found to have a peroxidase like activity which is not found in their monometallic counterparts. These nanostructures can be prepared easily in a short time, and have high catalytic activity. Apart from their use in immunoassay format, they can also be used further to detect peroxide containing chemicals under harsh conditions. These advantages easily overcome the shortcomings of natural enzymes. Moreover, they show a readily tunable catalytic activity by controlling the structure, composition, pH and temperature.

5.2 Preparation and characterization of bioconjugates

To develop a specific and sensitive immunoassay for small molecules, the crucial step is to generate good quality antibodies against the target. 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. The method used in the present study included modification of bensulfuron-methyl to generate carboxy group on the target moity followed by a carbodiimide activation method for carboxylated hapten to

link with carrier proteins, which ensured stable crosslinking of haptens with proteins which was characterized by fluorescence quenching, native PAGE and Mass spectrometry.

Nanoparticles and nanostructures were conjugated with antibodies, DNA library as well as with peptides to act as potential carriers. These nano-bioconjugates were characterized using Dynamic light scattering, UV-Vis spectrophotometer and Transmission electron microscopy.

5.3. Preperation and characterization of receptor molecules

In the development of DNA based receptor (aptamers), highly specific aptamers using ss-DNA libraries by nanoparticles mediated modified SELEX method were screened. The screened aptamers demonstrated higher specificity for hapten molecule. The subsequent nucleic acid based assay was developed that exhibited moderate sensitivity and specificity showing a detection limit of ~35 ng/mL. Further work is needed to increase the stability and sensitivity of the aptamers for their potential applications in ultra-sensitive biosensing platforms.

Antibodies were generated in mice and rabbit using both conventional as well as nanoparticles based approach. The generated antibodies showed very good sensitivity and selectivity against bensulfuron (IC_{50} ~ 5 ng/mL for IgG types anti-bensulfuron antibodies. Antibodies generated through nanoparticles as carrier route yielded higher titre than conventional route. More study is needed to ascertain the mechanism involved in nanoparticles mediated antibody generation.

5.4. Development of Biochemical Techniques

a) ELISA

Competitive immunoassay was performed using Rabbit anti-bensulfuron antibodies as immobilized capture antibodies. The sensitivity was determined by using antibody and free

hapten in competitive immunoassay format. Antibodies showed very high sensitivity with bensulfuron (IC_{50} equal to 5 ng/mL).

b) Fluoroimmuno assay using Pd@Au bimetallic nanostructures as peroxidase mimic

We have demonstrated a promising peroxidase like Pd@Au bimetallic nanostructures and their use in the development of a sensitive fluoro-immunoassay taking herbicide bensulfuron-methyl as model target. These nanostructures can be prepared easily in a short time, and have high catalytic activity. Apart from their use in immunoassay format, they can also be used further to detect peroxide containing chemicals under harsh conditions. These advantages easily overcome the shortcomings of natural enzymes. Moreover, they show a readily tunable catalytic activity by controlling the structure, composition, pH and temperature. The method presented here proved to be sensitive and inexpensive, as the overall cost for analysis remains very low due to use of small amount of nanoparticles. The proposed Pd@Au based immunoassay is very easy to perform and act as a sensible alternative to ELISA. Pd@Au nanostructures are robust and effective peroxidase mimics and may find promising applications in biocatalysis, diagnostics and bioassays.

c) Chemiluminescence assay using Pd@Au bimetallic nanostructures as peroxidase mimic

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 Pd@Au Nanostructures on chemiluminiscent intensity was measured by taking different size gold nanoparticles seed and Pd shells. It was shown that the CL intensity of 5 nm GNPs and 0.03 % Pd shell showed the highest reading as compared other 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 nanostructures.

Competitive inhibition using IgG bensulfuron-Pd@AuNS on microplate based inhibition assay was developed. Different concentrations of bensulfuron were prepared in the range of 0 to 1000ng/mL for the inhibition assay. The limit of detection was calculated to be around 0.5 ng/mL with IC_{50} value of anti-bensulfuron antibody.

d) Electrochemical stripping Voltammetry of gold ions based immunoassay

An enzyme free electrochemical immunoassay (EFEIA) based on gold nanoparticle tagged antibody is demonstrated for the detection of small molecule herbicide. The high sensitivity achieved is primarily because of dissolution of GNPs into large quantity of gold ions detected by anodic stripping voltammetry. High throughput screening of the large amount of samples is possible because of requirement of low volume of sample in microtitre plate. The approach can readily be used for rapid analysis of large number of assays of different analytes of environmental or clinical importance as a portable device with an array implanted electrodes in the microplate. High sensitivity for EFEIA (EC_{50}~ 6.7 pg mL^{-1}) in comparison to that of conventional ELISA (EC_{50}~ 4.97 ng mL^{-1}) was obtained._This Approach can be readily utilized for simultaneous screening of an array different analytes of environmental or clinical importance.

5.5 Future Perspectives

The high rate of population growth in the world requires high yields of crops form existing agricultural land. For high yields of food grains, the use of chemical fertilizers and pesticides has become indispensable. 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. Biosensors 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.

Despite the huge potential of biosensors, and the ever-increasing number of biosensors developed, commercially available biosensors are being applied to a restricted area of the potential market. In general, biosensors for environmental analysis have several limitations: sensitivity, response time, and lifetime, which should be improved for them to become a competitive analytical tool. The areas of development that are expected to have an impact in biosensor technology are: immobilization techniques, nanotechnology, miniaturization, and multisensor array determinations. There is a growing tendency towards miniaturization of analytical systems, since it allows the handling of low-volume samples, a reduction in reagent consumption and waste generation, and increases sample throughput. Taking advantage of miniaturization benefits, sensors and biosensors can become inexpensive and easy-to-handle analytical devices for fast, reliable measurements of chemical species. Development of sensors capable of determining several analytes simultaneously can represent an interesting tool in environmental monitoring and screening. This configuration allows the reduction in time and sample volume and other reagents required. However, a crucial aspect may be the production of new sensing elements easy to synthesize and with the capability to broaden the spectra of selectivity that can be reached by a biosensor. At present, the preparation and production in large scales of biomolecules such as enzymes or antibodies need an investment of time and knowledge. Synthetic peptides and MIPs are contemplated as promising alternatives overcoming the above-mentioned limitations. Unfortunately, the affinity accomplished by these synthetic receptors is still several orders of magnitude below that of the antibodies. Improvement in the affinity, specificity, and mass production of the molecular recognition components may ultimately dictate the success or failure of detection technologies. The possibility of tailor binding molecules with predefined properties, such as

selectivity, affinity, and stability, is one of the major aims for biotechnology. The development of advanced receptors will allow the analysis of complex real samples and in situ measurements resolving the responses from the analyte and from nonspecific background effects. Since scientific attention is currently being given to biotechnology, as this review has pointed out, the development of improved molecular recognition elements will be followed by a corresponding enhancement of the biosensor features.

From the above viewpoint, it is clear that the future of biosensors will rely on the success of emerging sophisticated micro and nanotechnologies, biochemistry, chemistry, thin-film physics, and electronics. To reach this goal, an important investment in research, expertise, and the necessary facilities is needed. However, as the world becomes more concerned about the impact that environmental contamination may cause on public health and the ecosystem, the demand for rapid detecting biosensors will only increase.

The analysis of complex matrices and of analytes difficult to determine by the actual analytical procedures (i.e., highly polar compounds), are progressively being approached by biosensors. However, there is still a lack of alternative biosensing systems for an important bunch of emerging contaminants such as bisphenol A, phtalates, and polybrominated compounds (used as flame retardants), veterinary and human medicines and personal care products (nutraceuticals, synthetic fragances, sun screen agents, etc.).