BIOMIMETIC AND OPTOELECTRONIC SENSORS FOR SELECTED ENVIROTOXIC MARKERS

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.....To My Beloved Parents

DECLARATION

I hereby declare that the Ph. D thesis entitled **"BIOMIMETIC AND OPTOELECTRONIC SENSORS FOR SELECTED ENVIROTOXIC MARKERS"** is an independent work carried out by me at the Chemical Sciences and Technology Division, National Institute for Interdisciplinary Science and Technology (NIIST), CSIR, Trivandrum, under the supervision of Dr. T. Prasada Rao and the same has not been submitted elsewhere for any other degree, diploma or title.

In keeping with the general practice of reporting the scientific observations, due acknowledgements has been made wherever the work described is based on the findings of other investigators.

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CERTIFICATE

This is to certify that the work embodied in the thesis entitled **"BIOMIMETIC AND OPTOELECTRONIC SENSORS FOR SELECTED ENVIROTOXIC MARKERS"** has been carried out by Mr. Prathish K. P under my supervision and guidance at the Chemical Sciences and Technology Division, National Institute for Interdisciplinary Science and Technology (NIIST), CSIR, Trivandrum and the same has not been submitted elsewhere for a degree.

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AAS	Atomic Absorption Spectroscopy
ACh	Acetylcholine
AChE	Acetyl cholinesterase
AIBN	Azo-bis isobutyronitrile
BEHS	Bis-2 ethyl hexyl sebacate xi
CBW	Chemical and biological weapons
CHEMFET	Chemically Modified Field Effect Transistor
CME	Chemically Modified Electrode
CTA	Cellulose Triacetate
CWC	Chemical Weapons Convention
DCP	Diethyl chlorophosphate
DOP	Dioctyl phthalate
EGDMA	Ethylene glycol dimethacrylate
FIA	Flow Injection Analysis
GCMS	Gas chromatograph coupled with mass detector
HEMA	2-Hydroxyethyl Methacrylate
HM	Heavy Metals
IPIM	Imprinted Polymer Inclusion Membrane
ISE	Ion Selective Electrode
ITO	Indium Tin Oxide

IUPAC	International Union of Pure and Applied Chemistry
LOD	Limit of Detection
MAA	Methacrylic Acid
MIP	Molecular Imprinted Polymer
MMS	Multimeasurand Solution
MPA	Methyl Phosphonic Acid
NIP	Non Imprinted Polymer
NIPIM	Non-imprinted Polymer Inclusion Membrane
NPOE	2-nitrophenyl octyl ether
OP	Organophosphates
PIP	Persistent Inorganic Pollutants
PVC	Polyvinyl Chloride
Rh 6G	Rhodamine 6G
RVD	Residual Vapor Detection Kit
SLE	Sequential Ligand Exchange
TCD	Three Color Detector paper
TEHP	Tris-2 ethyl hexyl phosphate
THF	Tetrahydrofuran
Tris	Tris–(hydroxymethyl)aminomethane
USEPA	United States Environmental Protection Agency
VA	4-Vinyl aniline
VP	4-Vinyl Pyridine
WMD	Weapons of Mass Destruction
WPDK	Water Poison Detection Kit

PREFACE

Environmental monitoring of air, water, and terrestrial zones for compounds that may pose a risk to human or ecosystem's health is a critical part of the efforts of scientists directed towards pollution prevention, industrial and agricultural waste regulation, and cleanup of hazardous waste sites. The high cost and slow turnaround times typically associated with the measurement of regulated pollutants clearly indicates a need for environmental screening and monitoring methods which are fast, portable, and cost-effective. Chemical and biological sensors have found their niche among modern analytical instruments when real-time determination of the concentration of specific sample constituents is required. Chemical sensors based on electrochemical (voltammetry, potentiometry) and optical transducers became an increasingly attractive tool for toxin monitoring, for quality control, and in medicinal and environmental chemistry. In addition to sensitivity, environmental analysis requires high selectivity and multianalyte capabilities owing to the complexity of the respective matrices. Molecular imprinting is a promising technology that confers specific molecular recognition capabilities to synthetic polymers. The binding sites that are generated during the imprinting process often have affinities and selectivities approaching those of antibody/antigen systems, and molecularly imprinted materials have therefore been dubbed 'antibody mimics'. MIPs compared with biosensors are stable, cost effective and easier synthetic protocols and can be applied to a diverse range of analytes from simple organic molecules or ions to macromolecules.

Another arena of cost effective and easy to handle sensors is optical sensors based on simple colorimetric principles. Ion association based self assembly of various species in aqueous media with a chromophoric receptor by means of intermolecular forces, including hydrogen bonding, π-π and hydrophobic interactions is attractive since not only it eliminates extra steps in synthesis, but it also allows for employment of combinatorial method: variation of dyes with the same ligand (or *vice versa*) may create libraries of ion-sensitive ensembles. Complex formation in a tri-component system metal cation (M^{n+})– anionic ligands (L) – dye cation (+) was expected to proceed in one of the two contrasting ways: **(i)** competitive complexation of R^+ and M^{n+} with L, which normally results in the dye displacement from its complex with anionic ligand by the metal ion, or **(ii)** assembly of a tri-component outer sphere complex ie. ion associates. Significant improvement of the sensitivity and selectivity, lowering of the detection limits, and decrease in the time taken for analysis can be achieved using the chemico-analytical properties of ion associates.

Chapter 1 gives a brief introduction on the need for developing MIP based potentiometric sensors for anionic and neutral analytes and its application potential. In addition, extensive literature survey carried out on the molecular imprinted polymer materials for the selective sensing of various analytes via potentiometric transducers has also been discussed. The chapter also emphasizes the need for developing sensors for the quick and selective sensing of organophosphorous pesticides and chemical warfare agents.

Chapter 2 deals with the synthesis of methyl phosphonic acid (MPA) imprinted polymer materials and subsequent design of polymer inclusion membranes for potentiometric sensing studies. Comparative evaluation of the efficiency of functional monomers viz. methacrylic acid and 4-vinyl pyridine has been done for effective sensing characteristics. The chapter also covers the study of influence of various parameters like plasticizer, MIP to PVC ratio, pH etc for optimum sensing. The application potential of the developed sensor has also been highlighted.

Synthesis and construction of MIP based potentioselctrode for a neutral analyte -phorate, an organophosphorus pesticide is described in **Chapter 3.** The noncovalent imprinting procedure and the influence of various parameters on the sensing characteristics have been emphasized. The application of the developed sensor in analyzing natural water samples has also been enumerated.

A comparative analytical evaluation of two imprinting strategies viz. Non-covalent and Semi-covalent imprinting for potentiometric sensing application has been done in **Chapter 4**. A reactive simulant of sarin, viz. diethyl chlorophosphate a potent nerve agent has been selected as the analyte for the studies. The chapter describes a one pot synthesis of ready to use MIP based potentiometric sensing membrane and its spectral and morphological characterization. Potentiometric sensing studies have been conducted using these membranes and applied for natural water sample analyses.

Chapter 5 gives an introduction to ion associate based optical molecular probes in aqueous media, especially ion associates of water soluble dyes with various anionic/cationic complexes and analytical applications based on their spectral characteristics have been highlighted. A brief discussion on the toxicity of heavy metals on the ecosystem and the urge for simultaneous or sequential multianalyte sensing has been provided. The chapter emphasizes the application potential of ion associates as optical molecular probes and tuning their properties using metal-ligand binary complexes.

Sequential multianalyte sensing of spectroscopically silent heavy metal toxins viz. Pb, Cd and Hg using the metal iodide induced aggregation of xanthene dyes is described in **Chapter 6**. The subtle variation in the stability constants of the metal iodide complexes of these metals were advantageously utilized for the sequential deaggregation process. The chapter discusses the various critical parameters like media, pH, concentration of iodide, xanthenes dyes, gelatin etc for obtaining the optimum sensing characteristics. Dual sensing capability of the optical molecular probes has also been studied. The developed sensing ensemble has been utilized for analyzing industrial and environmental samples.

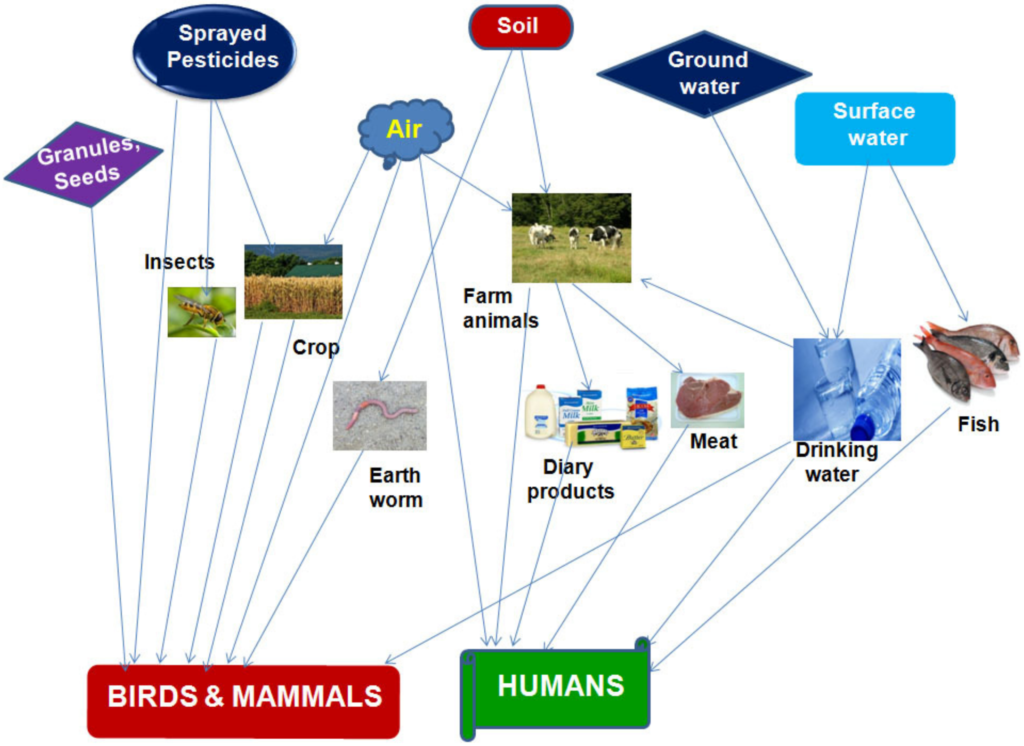
Chapter 7 describes brief summary and highlights the overall conclusions based on the results obtained.

Chapter 1

Molecular Imprinted Polymer Based Potentiometric Sensors: An Overview

1.1 INTRODUCTION

Environmental pollution by organic chemicals continues to be one of the world's leading challenges to sustainable development. Modern developed and developing countries utilize millions of synthetic organic compounds in their civilian, commercial, and defence sectors for an ever-expanding diversity of uses [Ariese *et al.* 2001]. Common applications include plastics, lubricants, refrigerants, fuels, solvents, preservatives, surfactants, dispersants and pesticides. As a result of widespread global usage coupled with improper handling practices, many of these organic compounds enter the environment and cause air, water and soil pollution. For example, pesticides and herbicides are applied directly to plants and soils, while accidental releases originates from spills, leaking pipes, underground storage tanks, waste dumps and waste repositories. Many pesticides are sprayed in large amounts with only 1% reaching the intended target. Some of these contaminants have long half-lives and thus persist to varying degrees in the environment. They migrate through large regions of soil until they reach water resources, where they may lead to ecological or human-health threat [Karr and Dudley, 1981]. Organisms, vegetation, animals and humans are affected by various chemicals through absorption, inhalation or ingestion. Scheme 1.1 shows the various pathways of bioaccumulation of these pesticide residues into the food chain [Vermeire *et al.* 2003]. These contaminants pose serious to fatal health hazards, such as asthma, birth defects and adversely affect the sustainability of life. Therefore, environmental monitoring is required to protect the public and the environment from possible organic toxins released into the air, soil and water.



Scheme 1.1 *Pathways of bioaccumulation of pesticides into the food chain*

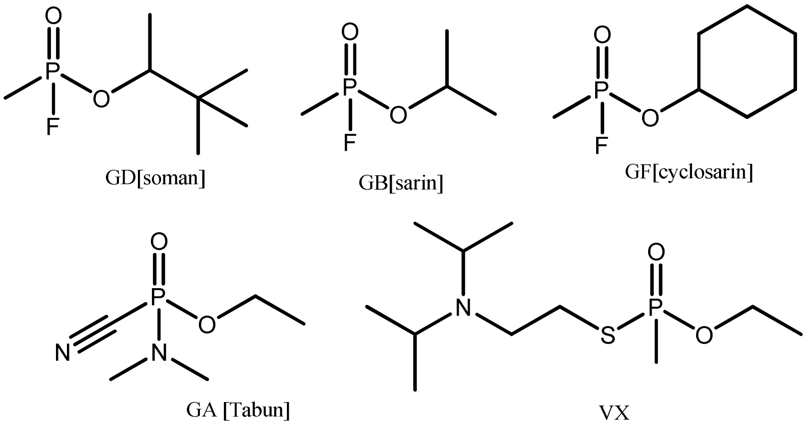
Pesticide contamination of ground water, which has emerged as an important environmental problem in past few decades, caused serious concern with respect to long-term and low-dose effects of pesticides on public health as well as non-target species of aquatic life [Sudo *et al*. 2002]. The use of excessive pesticides in agriculture may lead to contamination of surface and ground water by drift, runoff, drainage and leaching. Surface water contamination definitely have ecotoxicological effects on aquatic flora and fauna and human health if the water sources are used for public consumption [Forney and Davis, 1981; Mulla and Mian, 1981]. Surface water contamination usually depends on the agricultural season and does not last long, while ground water contamination has a strong inertia, which may cause continuous human exposure [Funari and Vighi, 1995]. Hence there is a need for continuous monitoring of surface and ground water contaminants.

The United States Environmental Protection Agency (USEPA) has imposed strict regulations on the concentrations of many environmental contaminants in air and water [Solomon and Huddle, 2002]. However, current monitoring methods for most organic contaminants are costly and time-intensive, and limitations in sampling and analytical techniques exists [Ho *et al.* 2005]. Thus, there is a great demand for development of quick, simple and reliable methods for the detection of organic pollutants.

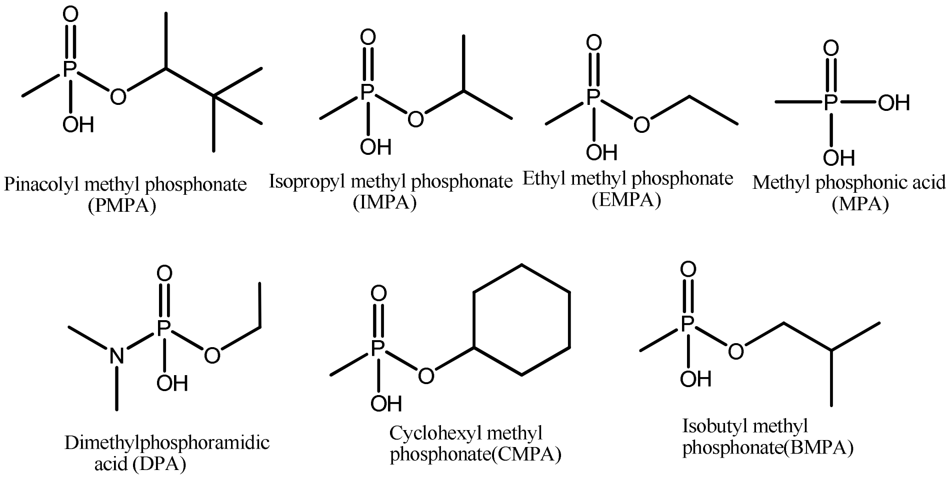
1.1.1 Organophosphorous Compounds

Many organophosphates (OPs) are highly neurotoxic because they disrupt the cholinesterase enzyme that regulates the neurotransmitter acetylcholine, which is needed for proper functioning of nervous system [Rosenberry, 1975; Zhang *et al.* 2001; Fennouh *et al.* 1997; Cremisini *et al.* 1995; Guerrieri *et al.* 2002]. There are atleast 13 types of OPs and hundreds of OP compounds in use, which are derivatives of phosphoric, phosphonic, or phosphinic acids [Gupta, 2006]. Most are used as pesticides, but a few have been used as chemical weapons. After the tragic events of September 11, 2001, the perceived threat of a chemical attack by terrorists has emphasized the need for early warning and continuous monitoring for taking effective countermeasures that can rapidly diagnose and mitigate the effects of highly toxic chemical exposures [Goozner *et al.* 2002; Rosenbloom *et al.* 2002].

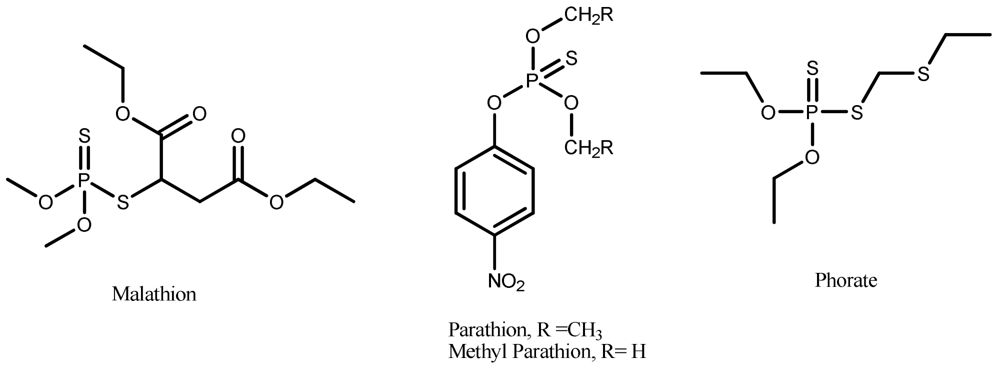
A) Chemical warfare agents (CWAs)



B) CWA simulants and degradation products



C) Organophosphorous Pesticides



Scheme 1.2 *Examples of organophosphorous chemical warfare agents, simulants, degradation products and pesticides*

Some of the common organophosphorous chemical warfare agents and pesticides are shown in Scheme. 1.2.

1.1.2 Pesticides

Pesticides are described as chemicals that kill or slow down the growth of undesirable organisms. Pesticides include herbicides, insecticides, fungicides and nematocides. Nowadays, it is believed that application of synthetic pesticides is one of the most effective methods for controlling insects that affect crop growth [Celic *et al.* 1995]. OP pesticides constitute the most widely used insecticides available today. This class of compounds has achieved enormous commercial success as a key component in the arsenal of agrichemicals, and is currently an integral element of modern agriculture across the globe. According to the EPA, about 70% of the insecticides in current use in the US are OP pesticides [Solomon and Huddle, 2002]. They were developed to replace organohalide pesticides in the late 1950's because OP pesticides are relatively easier to degrade via microbial or environmental processes. Unlike organohalide pesticides, the OP pesticides do not bioaccumulate due to their rapid breakdown in the environment and they are thus preferred over organohalides for insecticide and/or pesticide use.

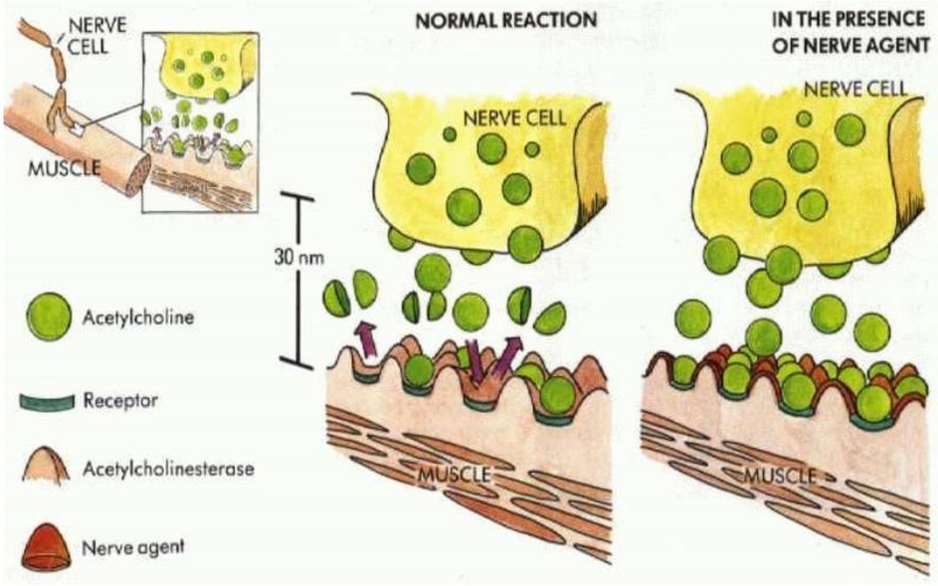
Although OP compounds are considered safer than organohalides, they are still highly neurotoxic to humans and in some cases their degradation products have the potential to be more toxic with chronic exposure [Vermeire *et al.* 2003]. OP compounds are efficiently absorbed by inhalation, ingestion and skin penetration. They are strong inhibitors of cholinesterase enzymes that function as neurotransmitters, including acetylcholinesterase, butylcholinesterase and pseudocholinesterase. These enzymes are inhibited by binding to the OP compounds. Upon binding, the OP compounds undergo hydrolysis leading to a stable phosphorylated and a largely unreacted enzyme. This inhibition results in the accumulation of acetylcholine at the neuron/neuron and neuron/muscle junctions or synapses thereby disrupting the normal nervous system functioning causing subsequent acute health risks.

1.1.3 Chemical warfare agents

Among the weapons of mass destruction (WMD), chemical warfare (CW) is probably one of the most brutal created by mankind. CW agents are extremely toxic synthetic chemicals that can be dispersed as a gas, liquid or aerosol or as agents adsorbed to particles to become a powder. These CW agents have either lethal or incapacitating effects on humans [Aas, 2003]. They differ from explosive chemicals in which the destructive effects are caused by shear force and are localized. Thousands of toxic substances are known, but only some of them are considered as CW agents based on their characteristics, viz. high toxicity, imperceptibility to senses and rapidity of action after dissemination and persistency, and are listed as scheduled chemicals in the chemical weapons convention (CWC) [Hooijschuur *et al.* 2002]. According to the CWC, chemical weapons are defined as toxic chemicals and their precursors, munitions and devices, and any equipment specifically designed for use directly in connection with such weapons.

The major reasons for the production and use of such weapons are manifold. First, chemical weapons are cost-effective, particularly when used against concentrated forces or populations. Second, they may be used at lower levels of concentration with an aim to cause panic and disorder among civilians. In Tokyo subway attack, a simple plastic bag containing the CW agent sarin was kept on an underground train, allegedly piercing it with umbrella tips before the cults escaped [Tu, 2000]. Therefore, intentional release of such agents during national or international events can be easily achieved by transporting them in the form of water bottles, cold drink cans, ampoules or even pens, etc. to the site.

The mode of action of nerve agents is well documented in the literature [Somani, 1992; Bajgar *et al.* 2009; Bajgar, 2004; 2005]. Nerve agents exert their biological effects by irreversibly inhibiting the enzyme acetyl cholinesterase (AChE). This enzyme is responsible for hydrolyzing acetylcholine (ACh), a neurotransmitter liberated at the nerve synapse, nerve muscle (neuromuscular) junction and nerve gland junction. In a normal individual, a small quantity of ACh is continuously liberated and hydrolyzed by AChE. Inhibition of AChE causes accumulation of ACh, leading to overexcitation or paralysis. As soon as the nerve agents enter the system, symptoms of poisoning appears. The mode of action of nerve agents are shown in Scheme 1.3.



Scheme 1.3 *Mode of action of nerve agents*

The effects of nerve agents are the result of the action on the muscarinic and nicotinic receptors within the central nervous system. They include constriction of the pupil (meiosis), increased production of saliva, running nose, increased perspiration, urination, defecation, bronchosecretion, bronchoconstriction, decreased heart rate and blood pressure, muscular twitches and cramps, cardiac arrhythmias, tremors and convulsions. The most critical effects are paralysis of the respiratory muscles and inhibition of the respiratory centre. Ultimately, death results due to respiratory paralysis. If the concentration of the nerve agent is high, death is immediate**.**

1.2. EXISTING DETECTION TECHNIQUES

Analysis of OPs in environmental and biological samples is routinely carried out using analytical techniques, such as gas or liquid chromatography and mass spectrometry [Sherma, 1993]. Such analysis is generally performed at centralized laboratories, requiring extensive labor and analytical resources, and often results in a lengthy turnaround time. These analytical methods have a number of disadvantages that limit their applications primarily to laboratory settings and prohibit their use for rapid analyses under field conditions. Definitive identification of an agent can be carried out on-site in a mobile analytical laboratory or in an off-site laboratory, and this will generally take many hours. Gas chromatograph coupled with mass detector (GCMS) fitted on a vehicle can be used for unambiguous detection of most of the organic compounds, including CW agents, at very low concentrations [Smith, 2002]. Photograph 1a shows Mobile lab donated by Agilent Technologies to New York police department (NYPD) that is specifically designed to assist with on-the-spot collection and analysis of suspected chemical and biological agents. Contaminated samples are received in the rear of the van, prepared under an in-house fume hood, and then transferred to a clean room in the front of the van. Photograph 1b shows the interior of mobile lab showing Agilent 6890 GC with mass-selective detector [Smith, 2002].

However, this hyphenated technique is complex, requires skilled operators, is difficult to maintain and does not provide much advantage in comparison with off-site analysis, especially when chemical incident occurs at remote site where the mobile lab cannot approach easily.

Photograph 1a *Mobile lab donated by Agilent Technologies to NYPD.* ***b)*** *Interior of mobile lab showing Agilent 6890 GC with mass-selective detector [Smith, 2002].*

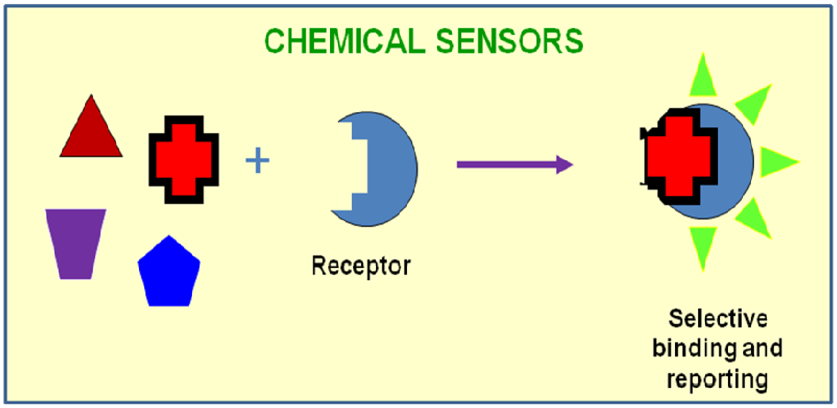
The rapid identification and qualitative and quantitative determination of the unknown agent is necessary for the selection of adequate protective measures (protective masks and clothing as well as medical treatment), the mapping of contamination area and decontamination procedures [Makas and Troshkov, 2004; Murray and Southard, 2002; Gripstad, 1992; Murray and Lawrence, 2005; Baumbach and Eiceman, 1999; Collins and Lee, 2002; Sun and Ong, 2005]. For onsite verification, especially involving CW agents, several handheld detection devices are available, including Three Color Detector (TCD) paper, Residual Vapor Detection (RVD) Kit, Water Poison Detection Kit (WPDK) and Chemical Agent Monitors. These are simpler, often cheaper and more sensitive, but cannot be used for continuous monitoring of the air or water quality and pose several limitations, such as low specificity and inability to detect all CW agents. The current detection technologies for CWAs can be divided into point and standoff detectors. Point detectors employ techniques such as Ion Mobility Spectrometry, Flame Photometry, Mass Spectrometry and Photoacoustic Infrared Spectroscopy. Each of these, sample the air in the immediate vicinity of the detector [Xie *et al.* 1999]. In contrast, standoff detectors use infrared remote sensing technologies from a significant distance. However, the effects of humidity, temperature and composition of the air may influence the detector response and often render false results.

Biological methods such as immunoassay and inhibition studies, which monitor acetylcholinesterase activity shows great potential for the detection of CWAs but suffer from extensive sample handling procedures and are at present not suitable for real-time monitoring [Mulchandani *et al.* 1999; Guodong and Lin, 2006]. The methods that meet the needs of real-time analysis, such as fiber-optic sensors [Jenkins *et al.* 1999], surface acoustic wave devices [Nieuwenhuizen and Harteveld, 1997], or microbial biosensors [Mulchandani *et al.* 2001] often lack selectivity and do not have an optimal alarm ratio.

The rapid identification of chemical and biological weapons (CBW) outside the laboratory poses another daunting challenge, frequently likened to the proverbial “needle in a haystack” problem: the agents in trace amounts must be detected in complex backgrounds (soil, seawater, bodily fluids etc.). These backgrounds contain chemicals that may inhibit the sensor and/or "clutter" that can confound agent detection. One portable analytical tool that is already making an impact in the war against weapons of mass destruction or pesticide monitoring is the chemical sensor. [Demirev *et al* .2005]. Civilians would be more receptive to smaller, lapel-size flashing sensors or smoke-alarm size air-quality detectors that could give more immediate results. Real-world backgrounds are diverse and highly variable and can impact sensor performance unpredictably (e.g., specificity and limit of detection).

1.3 SENSORS

Biosensors and chemical sensors have attracted considerable attention in the field of modern analytical chemistry because of their extreme sensitivities and specificities. They can be successfully employed for the analysis of materials in different fields such as clinical diagnostics, environmental control, food analysis and drug screening. The central part of such sensors is the chemical or biological molecular recognition element, also named as, receptors, which can recognize and bind the target analyte specifically. For biosensors, these recognition elements include antibodies, enzymes, DNA probes and cells [Suryanarayanan *et al.* 2010; Nakano *et al.* 1998; Tang *et al.* 2010]. The receptors are in close contact with transducers that converts the recognition between sensing (chemical/ biological) element and the target in to quantifiable signal. A schematic representation of the working of chemical sensors is shown in Scheme 1.4.



Scheme 1.4 *Principle of working of chemical sensors*

Biological recognition systems—though extremely useful—still exhibit limited thermal, mechanical and long-term stabilities, which makes the development of artificial materials a highly interesting research task. Moreover they have only limited applications in chemical assays as they involve expensive extraction and need highly controlled environment process for the production of antigen etc. Chemical sensors can be defined as ‘‘a small device, that as a result of a chemical interaction or process between the analyte and the sensor device, transforms chemical or biochemical information of a quantitative or qualitative type into an analytically useful signal’’. [Stetter *et al.* 2003] Furthermore, sensors for environmental samples usually require substantially increased selectivity and ruggedness because of the complex and diverse nature of sample matrices. Compared with established analytical strategies, the main focus of these novel developments has been to improve the analysis time, ideally leading to real-time results, and to cut analysis costs by reducing both device prices and operator time.

1.4 CLASSIFICATION OF CHEMICAL SENSORS

In a sensor device, a sensing material is applied onto a suitable physical transducer to convert a change in a property of sensing material into a suitable physical signal. The energy transduction principles that have been employed for chemical and biological sensing involve radiant, electrical, mechanical and thermal types of energy. Specific sensing concepts are further implemented with each energy transduction. Sensors based on radiant energy of transduction can employ intensity, wavelength, polarization, phase, or time resolution detection. Sensors based on electrical energy of transduction can employ conductometric, potentiometric or amperometric detection. Sensors based on mechanical energy of transduction can employ gravimetric or viscoelastic detection. Sensors based on thermal energy of transduction can employ calorimetric or pyroelectric detection [Potyrailo and Mirsky, 2008]. Hence, chemical sensors may be classified into four general categories such as optical, piezoelectric, electrochemical and thermal on the basis of their transduction [Bobacka *et al.* 2003].

1.4.1 Electrochemical Sensors

Electrochemical sensors are the oldest and most widely available group in the solid-state chemical sensor field. Of the above mentioned different transducers, electrochemical transducers become very popular due to ease of measurement and the availability of instrumentation. Moreover, they are found to be effective in offering good limits of detection (LOD) at low cost with the possibility of easy miniaturization and automation. In addition, since aqueous environments are typical of environmental samples, electrochemical approaches attract a particular amount of attention. They have a leading position among the presently available sensors that have reached commercial stage and found a vast range of important applications in the fields of clinical, industrial, environmental and agricultural analyses.

Many solid-state electrochemical sensors have been commercialized, such as glucose monitors for diabetes and ion sensors for blood electrolytes. This type of transduction is especially attractive with a view to making readily available a range of small devices in relevant applications such as biomarkers in clinical chemistry, environmental control in the field, on-line quality control in the pharmaceutical industry or detection of food fraud. Chart 1.1 depicts the various inherent advantages of electrochemical sensors over other transducers.

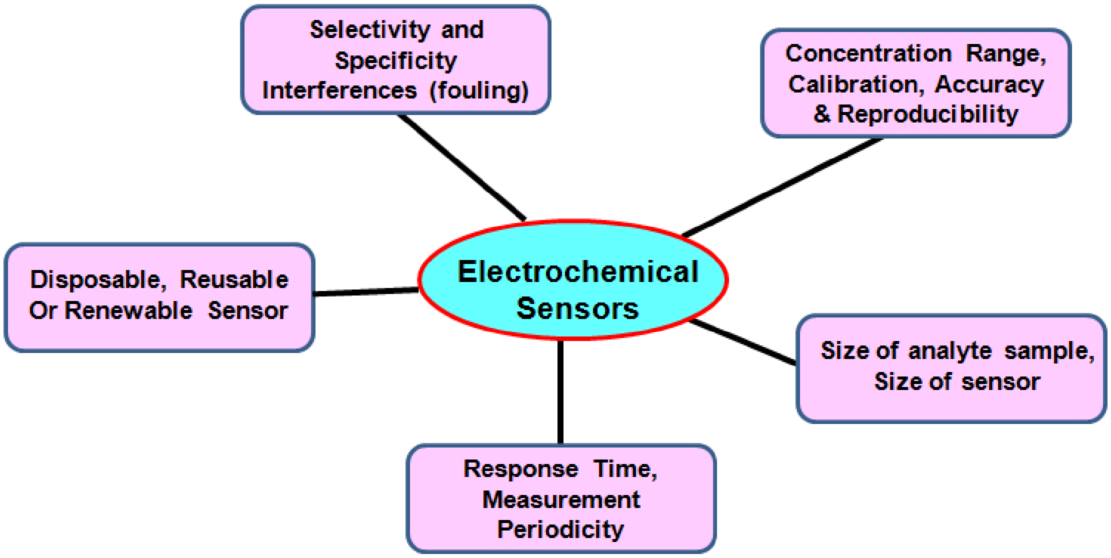


Chart 1.1 *Important aspects for choosing electrochemical sensors for environmental monitoring*

1.4.1.1 Types of electrochemical transduction

Electrochemical sensors are classified as potentiometric, conductometric and voltammetric sensors based upon their analytical principles of operation [Blanco-Lopez *et al.* 2004]. Chart 1.2.shows the classification of various types of electrochemical sensors based on transduction principle.

Potentiometric sensors measure an equilibrium potential difference between a sensing electrode and a reference electrode. Ideally, there is no current flow through the electrodes at equilibrium. In general, the potential difference shows a linear relationship with the logarithm of the activity of the analyte, as in the Nernst equation.

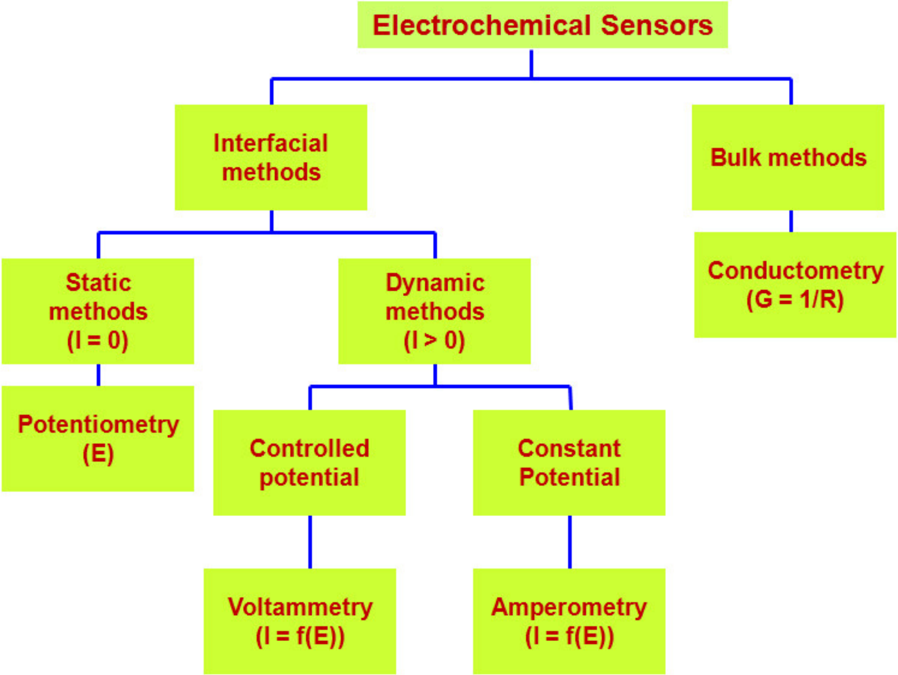


Chart 1.2 *Classification of electrochemical sensors based on transduction principle*

Conductometric sensors quantitate the changes of electrical properties between two electrodes. Resistive sensors measure the resistivity change due to chemical reactions, while capacitive sensors detect the capacitance change due to a dielectric-constant modification. Voltammetric sensors measure the current from the charge transport of an electrochemical reaction on a sensing (working) electrode when a varying potential or a constant potential (amperometric detection) is applied between the working electrode and the solution. Voltammetric sensors use an auxiliary (counter) electrode to control the solution potential and as an electron source or sink for the counter reaction to the one at the working electrode. For more stable analysis, most voltammetric sensors also use a nonpolarizable reference electrode to monitor the solution potential [Bakker and Pretsch, 2005].

1.5 POTENTIOMETRIC SENSORS

Potentiometry is based on creating a potential difference across a membrane placed between two solutions with charged species of different activity. As explained, potentiometry gives information about the ion activity. By contrast, voltammetric techniques detect analytes that have a sufficiently high electrochemical turnover rate at the electrode. Complexes that are chemically inert, or analytes that are bound to suspended particles, are normally not detectable. Voltammetric techniques, therefore, yield information on the concentration of chemically available (labile) analytes. However, atomic spectroscopic methods atomize the entire sample and do not distinguish between the different forms of the analyte. They yield the total concentration of the analyte of interest.

In a potentiometric chemical sensor, a synthetic receptor molecule is immobilised in a polymeric membrane. The presence of ionophore molecules is responsible for chemical sensing and signal generation at the electrochemical interface of a potentiometric sensor [Dybko and Wroblewski, 2002]. The first step in the design of a potentiometric sensor involves selection of an appropriate ionophore (electrically neutral or charged). In many cases this leads to the synthesis of a new ionophore structure dedicated to the ion to be measured. A new ionophore should exhibit high selectivity towards target ion and sufficient lipophilicity to ensure durable electrochemical interface. One side of the membrane is in contact with the studied analyte solution. The reversible exchange of ions between the analyte solution and the membrane phase (and the complexation reactions in the membrane) causes the formation of the membrane potential i.e. signal generation [Morf, 1981]. The potential difference across the selective membrane depends on the molar free analyte concentration. The change in potential is related to concentration in a logarithmic manner. The Nernst equation relates potential difference at the interface to the activities of species i in sample phases (s) and in the electrode phase (β):

$$E = E^0 + \frac{RT}{Z_i F} \ln \frac{a_i^s}{a_i^\beta}$$

where E^0 is the standard electrode potential of the sensor electrode; a_i is the activity of the ion, R is the universal gas constant; T is the absolute temperature; F is the Faraday constant; Z_i is the valency of the ion.

In the case of molecular recognition of an ion, the receptor is capable of extracting ions from an aqueous solution and acts as an ionophore or an ion-carrier. The complexation of the analyte ion by the ionophore results in the charge separation at the boundary of the solution/membrane interface. The transfer of the ion complexes through aqueous solution/membrane boundary occurs due to relatively high potential energy of dipole-ion interactions in a lipophilic membrane phase.

1.6 MODERN DIRECTIONS OF POTENTIOSELECTRODES

The overwhelming success with optical sensors in reaching low detection limits down to subnanomolar levels [Morf *et al.* 1990] put into question the unappealing detection limit of higher than micromolar levels observed with corresponding potentioselectrodes based on very same materials [Buhlmann *et al.* 1998]. In ideal cases (high sample concentrations) where concentration polarization in the aqueous phase boundary can be neglected, the membrane selectivity dictates the detection limit of potentioselectrodes [Buck and Lindner, 1994; Bakker *et al.* 1993]. It is to be mentioned here that IUPAC definition of detection limit for potentioselectrodes is defined as cross-section of extrapolated segments of the calibration curve [Buck and Lindner, 1994; Guilbault *et al.* 1976]. This does not correspond to that of all other analytical methods [Currie, 1995] (also by IUPAC), for which the lower detection limit is expressed in terms of the signal in the absence of analyte and noise. Understanding and eliminating the undefined zero current ion fluxes from the membrane into the sample solution, replacing the traditional internal filling solution of the membrane by solid contact, design of pulstrodes, miniaturization to micro and submicro electrodes, back side calibration potentiometry and implantable sensors are some of the approaches resulted in resurgence of potentioselectrodes [Sokalski *et al.*1997; 1999; Radu *et al.* 2007; Bakker and Chumbimuni-Torres, 2008].

From the above discussion, we can realise that there is a need for developing chemical sensors having better stabilities and cost effectiveness than biosensors coupled with sensitivities and selectivities comparable to that of bio receptors for routine environmental monitoring. Among the various types of electrochemical sensors, potentiometric sensors are superior in terms of portability and cost factor and several innovative approaches have improved its sensitivity remarkably during the past decade. One of the bottle necks of conventional potentioselectrodes is the selectivity. This aspect had not been addressed adequately and molecular imprinted polymers can be a facile alternative for selective molecular recognition. In order to replace natural systems, artificial receptors are gaining particular importance in recent years and even they mimic the recognition properties of biological materials (biomimetics). Methods based on this approach often make use of principles derived from supramolecular chemistry, which is one of the driving forces of nature. Molecularly Imprinted Polymers (MIPs) represent one such artificial receptor often dubbed as “artificial antibodies” and offer one of the first fully artificial, robust yet highly selective materials [Sellergren, 2001]. In recent years, MIPs have been discussed more generally as a special type and format of receptor design and structure, within the wider chemical world of supramolecular chemistry [Liu *et al.* 2007; Bossi *et al.* 2007; Qiao *et al.* 2006]. Consequently more of the advances in self-assembly and molecular recognition have been explored and incorporated into MIP design and synthesis.

1.7 MOLECULARLY IMPRINTED POLYMERS

Molecularly imprinted polymers (MIPs) are synthetic materials designed by emulating the way the immune system generates the selective complexants known as antibodies. Specifically, MIPs trace their origin to suppositions about the operation of the human immune system by Stuart Mudd in the 1930s and Linus Pauling in the 1940s. Mudd’s contribution to understanding the immune system was to propose the idea of complementary structures [Mudd, 1932]. This supposition states that the reason that a specific antibody attacks a specific target or “antigen” is because the shape of antibody provides an excellent fitting cavity for the shape of antigen. The description is very similar to the “lock and key” analogy used to explain the action of enzymes, the molecules responsible for hastening and directing biochemical reactions. Enzymes form a lock for a particular chemical key to fit, and as the “key” is turned, the enzyme directs and hastens the production of desired products from the chemical target.

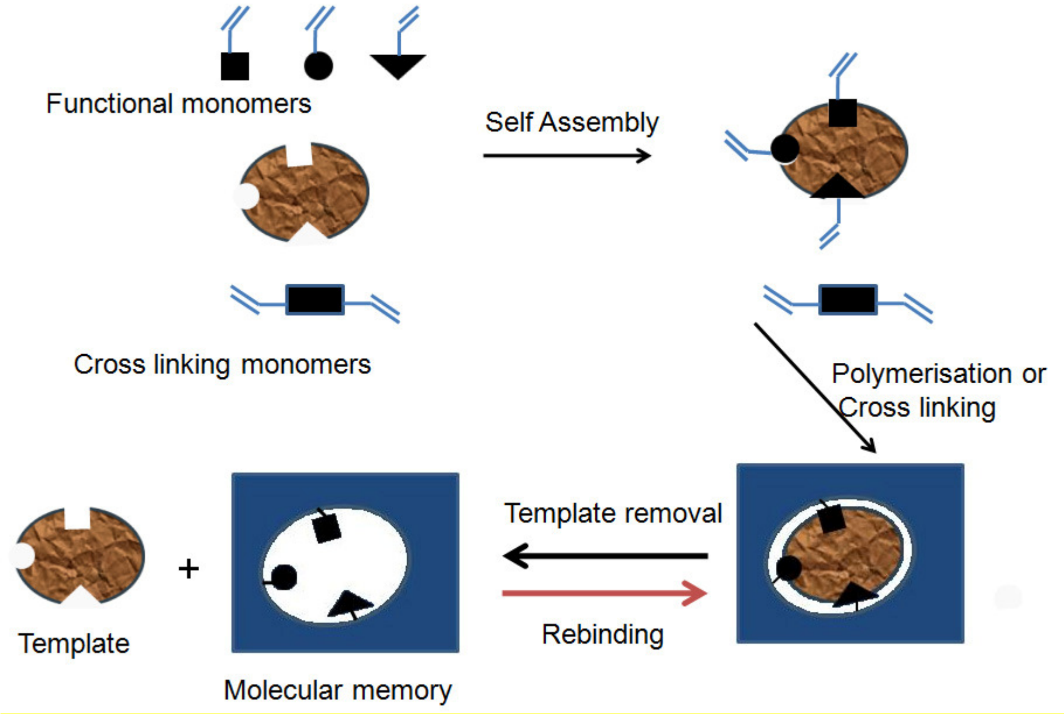
Pauling’s contribution to the development of MIPs was to explain the source of the complementary shape exhibited by antibodies [Pauling, 1940]. He postulated how an otherwise nonspecific antibody molecule could be reorganized into a specific binding molecule. He reasoned that shape specificity was obtained by using a target antigen to arrange the complementary shape of an antibody. Thus, a nonspecific molecule can be shaped to the contours of a specific target, and when the target is removed, the shape is maintained to give the antibody a propensity to rebind the antigen. This process is known as molecular imprinting or "templating." Molecular imprinting has been defined as: "The construction of template selective recognition sites in synthetic polymers where a template (atom, ion, molecule, complex or a molecular, ionic or macromolecular assembly, including microorganisms) is employed in order to facilitate recognition site formation during the covalent assembly of the bulk phase by a polymerization or polycondensation process, with subsequent removal of some or all of the template being necessary for recognition to occur in the spaces vacated by templating species" [Alexander *et al.* 2006].

The early work in molecular imprinting was based on silica gels, [Dickey, 1949; 1955] which showed some retention or memory of structure but were limited in application. The synthesis and characterization of molecularly imprinted organic polymers were first reported by Wulff and Sarhan [1972] from Dusseldorf University for sugar derivatives. Their investigations, and later those of Shea and coworkers, [1978; 1980] and Damen and Neckers [1973; 1980a; 1980b] indicated that polymers with specific cavities can be produced. Thereafter, different kind of substances such as sugars, amino acids, peptides, drugs, steroids, metals ions, dyes etc., have been imprinted and studied successfully.

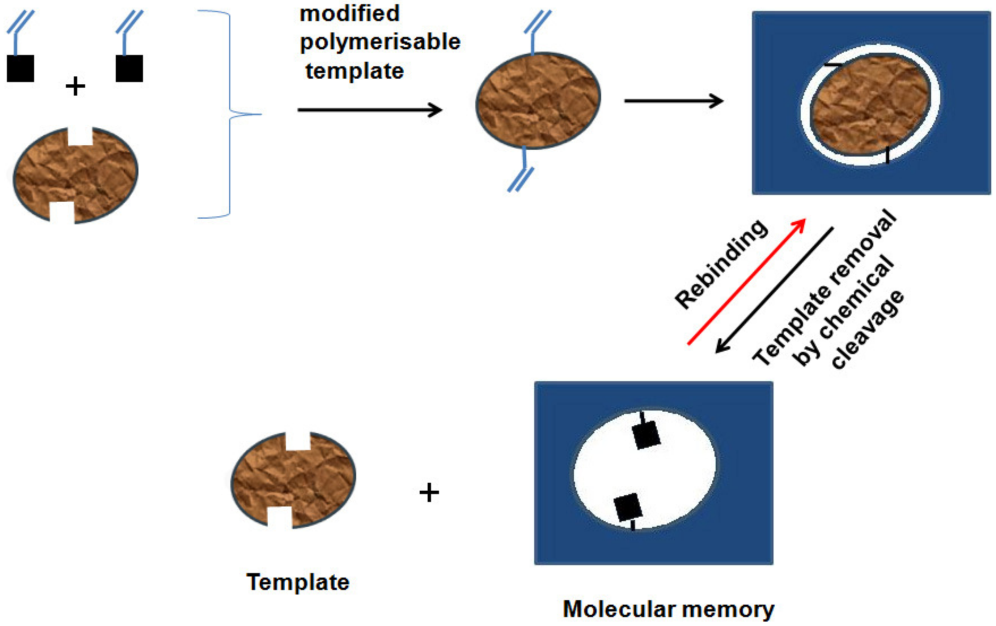
In MIP synthesis, the various components, template, functional and crosslinking monomers are mixed in solution prior to polymerization. This solution is commonly referred to as the prepolymerization mixture. The functional monomers form a complex with the target molecule using covalent or non-covalent interactions in the prepolymer mixture. The use of covalently bound target molecules was pioneered by Wulff *et al.* [1972; 1973] while the non-covalent approach to imprinting was pioneered by Mosbach [Arshady and Mosbach, 1981; Andersson *et al.* 1984]. The monomer contains polymerizable double bonds, which can react with crosslinking monomers (and other monomers) to create a polymer network. Once polymerized, the complex is postulated to be held into place by the cross-linking monomers. The template molecule can then be removed from the polymer leaving behind an imprinted site that is complementary in size, shape, and functionality to the target molecule. The polymer now has sites that are capable of recognizing and rebinding the target molecule with high specificity.

A representation of different strategies of molecular imprinting can be seen in Scheme1.5. Although the notion of combining the advantages of covalent bonds during the imprinting step with those of non-covalent interactions for rebinding was present in earlier work by Wulff et al. [1972; 1973; Lauer *et al.* 1985], the first example of using exclusively covalent interactions for the imprinting step and non-covalent ones for rebinding was executed by Sellergren and Andersson [1990]. Polymerizable groups were linked by ester bonds, eventhough the target molecule, p-aminophenylalanine ethyl ester, was necessarily different compared with imprinted template. Hence the rebinding process occurs via non-covalent interactions. This changed completely in 1994, when Whitcombe and coworkers [1994; 1995] significantly refined the semi-covalent approach, outlining a concept based on a small covalent fragment as space holder ("sacrificial spacer approach").

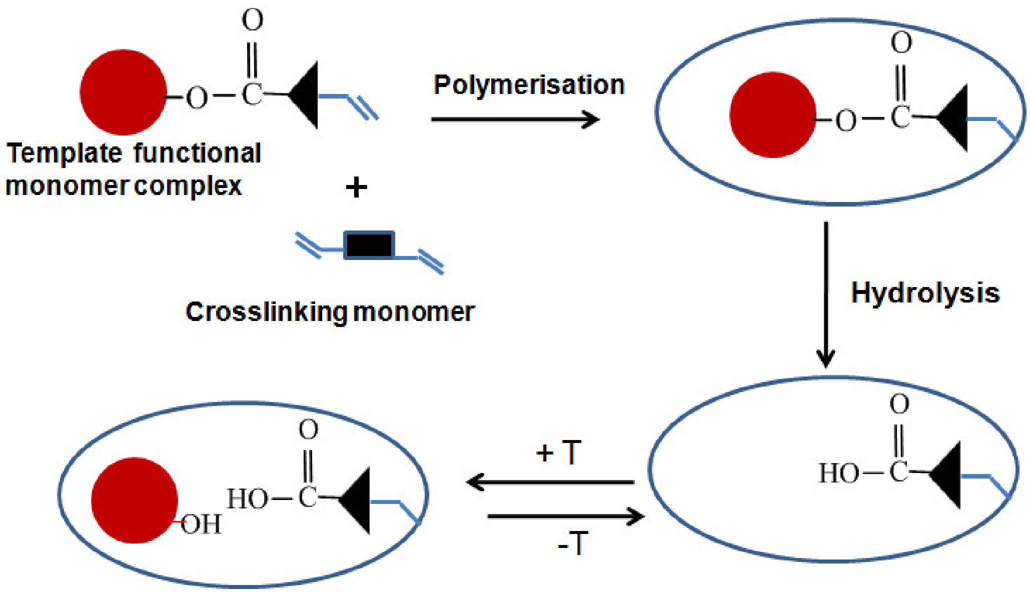
A. Non-Covalent Imprinting



B. Covalent Imprinting



C. Semi-covalent imprinting



Scheme 1.5 *Different strategies of imprinting (A) Non-covalent (B) Covalent (C) Semi-covalent*

With Sacrificial spacer strategy, template and a binding site are covalently linked with a cleavable spacer designed to reveal complementary functional groups upon removal, closely following the geometry of the covalent juncture for improved complementarity. The semi-covalent approach is an attempt to synergise the advantages of the covalent methodology (strict control of functional group location, more uniform distribution) with that of the non-covalent one (reduced kinetic restriction upon rebinding).

To be useful, Wulff and Sarhan [1972] indicated that these polymers must exhibit the following characteristics:

- The cavity geometry and the binding group arrangement should be preserved after the removal of the template molecule. This may be accomplished by high degrees of cross-linking, that is, use of a structural monomer that links chains together.

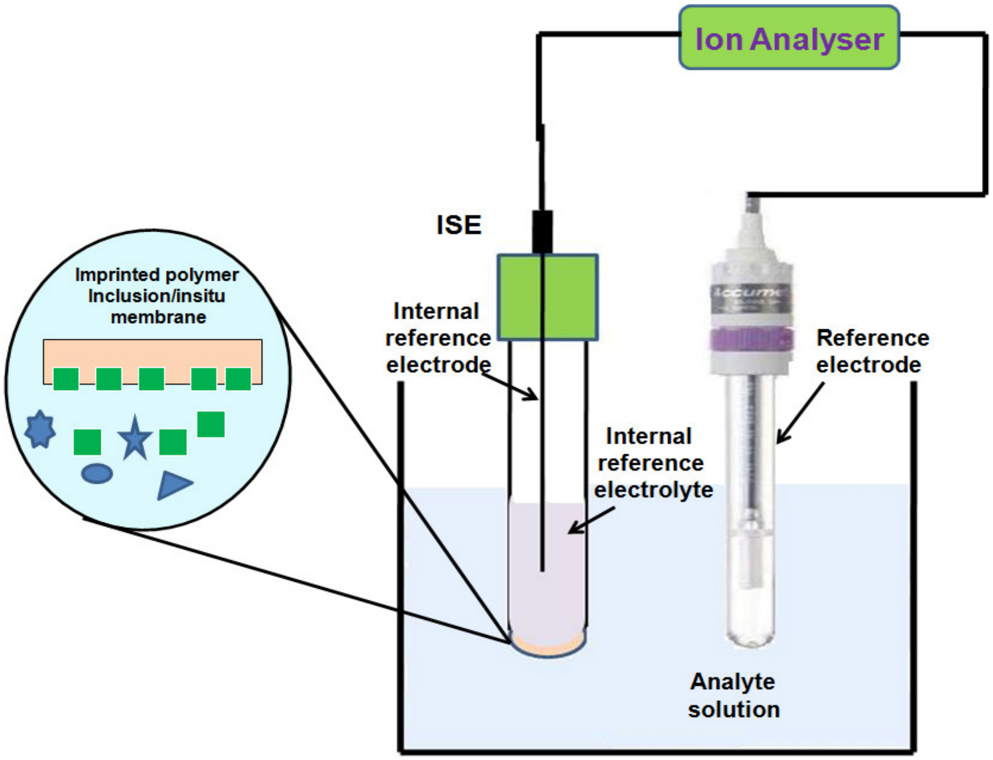
- The cavities must retain enough flexibility to permit rapid uptake and release of the template molecule. This property decreases with increasing cross-linking, so a balance must be sought.
- A large fraction of the template molecules should be removable after polymerization. This implies good accessibility of the cavities, which requires polymers with large surface areas.
- The polymeric materials should be chemically and mechanically stable to permit regeneration and reuse.

The recognition processes (host-guest interactions) of any analyte occurring in the receptor part govern the selectivity of the chemical sensors. Analyte recognition is based on selective complexation reaction of a guest molecule and synthetic or biological molecules [Gopel *et al.* 1989; Janata, 1989; Spichiger-Keller, 1998]. The target chemical components include ions as well as neutral molecules. The driving force of these reversible processes can be ion-dipole, dipole-dipole and hydrogen bond interactions. In certain cases, the analyte recognition process can be based on the formation of covalent bonds. The recognition of the guest molecules is achieved by the combination of complementary cavity shape and appropriate arrangement of the binding sites (functional groups) of the receptor (key-lock configuration). Due to the presence of shape and size selective cavities with complementary functional groups tailor made in the matrix, MIPs have high selectivity and affinity constants when compared to naturally occurring systems for sensor applications. They are very stable and robust, and are resistant to degradation in extreme environments such as in acids and bases or at high temperatures and pressures. Moreover, these materials are much cheaper than their counterpart and can be stored in a dry state at room temperature for long periods of time.

1.7.1 MIP based Potentioselectrodes

The two representative modes of signal transduction that can be induced by host-guest complexation at surface are membrane potential and membrane permeability changes. The former principle is extensively exploited for design and development of potentiometric sensors. Molecular imprinted polymers have evolved as tailor made molecular recognition domains and are successfully applied for the design of sensing membranes stimulated via surface potential generation. Rao and Kala [2008] have reviewed the design and development of biomimetic potentioselectrodes for inorganic and organic guests. For the development of MIP based potentiometric sensors, it is important to note that the creation of membrane potential does not require the template to be completely extracted from the membrane. This is an advantage, because extraction of the template to leave recognition sites ready for binding is very often a source of uncertainty at the determination or a sensitivity-limiting factor. Another unique feature of potentiometry is that species do not have to diffuse through the membrane, so there are no size restrictions on the template compound [Blanco-Lo´ pez *et al.* 2004]. Despite all these advantages, this type of transduction has not been explored much and we found only few reports.

MIP based potentiometric sensors are constructed by dispersing leached MIP particles in a PVC matrix along with a suitable plasticizer to form an imprinted polymer inclusion membrane (IPIM). The prepared sensing membrane is then attached to a glass tube filled with internal electrolyte and electrode to form the indicator electrode. The potential is measured on serial addition of analyte against a standard reference electrode. The target analytes selectively binds in the molecular recognition cavities in the IPIM sensor and the binding event generates a potential change which is measured using an ion analyzer. A schematic representation of the construction of an MIP based potentioselectrode is depicted in scheme 1.6.



Scheme 1.6 *Construction of MIP based Potentioselectrodes*

Other formats of MIP based potentioselectrodes include custom made MIP membranes prepared by in situ polymerisation, where “container defined” imprinted membranes are tailor made and after leaching can be directly integrated with transducer thereby eliminating an additional polymer support of PVC or CTA [Merkoci and

Alegret, 2002], thus widening the scope of imprinted polymers as ready to use molecular recognition substrates. Electrochemical signal transductions are based on molecular recognition occurring at membrane/aqueous solution interfaces, which frequently involve chemical processes that are quite different from those occurring in a solution bulk.

Odashima [1998] reviewed the trends in molecular recognition involving membrane potential changes induced by host-guest complexation with inorganic and organic guests. The review elaborates membrane potential changes due to inorganic guests, discrimination of organic guests based on polar & non polar structures and the membrane potential changes induced by neutral molecules. The following account highlights the developments in the field of MIP based potentioselectrodes for inorganic and organic guests. Table 1.1 summarizes essential features of MIP based potentioselectrodes reported in literature (since 2007) broadly falling under above two categories. Reports prior to 2007 are given in the review from our group. [Rao and Kala, 2008].

1.7.2 Membrane potential changes by inorganic guests

Mosbach, one of the pioneers of molecular imprinted polymers and his coworkers reported the first MIP based ion selective electrode for Ca (II) and Mg (II) ions [Rosatzin *et al.* 1991]. The imprinting process enabled better selectivity for Ca (II) by factors of 6 and 1.7 on Ca (II) binding using Ca (II) and Mg (II) imprinted polymer materials over non imprinted polymer particles. Further studies on the development of potential generation at the membrane solution interface using imprinted polymers for sensing inorganic cations were done by Prof.Murray’s Group of John Hopkins University.

Table 1.1 *MIP based potentioselectrodes designed since 2007*

SL.No	Template/ Analyte	Mode of Imprinting	Calibration Range (mol L^{-1})	Slope for decade change (mV/s)	LOD	Reference
1	Carcino embryonic antigen(CEA), Human amylase, Poliovirus	Non-Covalent (Surface imprinting)	2.5–250 ng/mL --- ---	-- -- --	-- -- --	Wang *et al.* 2010
4	Promethazine	Non-Covalent	5.0×10^{-7} -1.0×10^{-1}	31.2±1.0	1.0×10^{-7}	Alizadeh and Akhoundian, 2010
5	Melamine	Non-Covalent	5.0×10^{-6} -1.0×10^{-2}	54	1.6×10^{-6}	Liang *et al.* 2009
6	Quinine	Non-Covalent	4.0 x10^{-6} – 1.0 x10^{-2}	61.3 – 55.7	1.2 x10^{-6}	Kamel and Sayourb, 2009
7	Haemoglobin	Non-Covalent (Surface imprinting)	40 – 300 µg/mL	---	--	Wang *et al.* 2008
8	Myoglobin	Non-Covalent (Surface imprinting)	40- 250 µg/mL	---	---	Wang *et al.* 2008
9	Hydroxyzine	Non-Covalent	1.0 x10^{-6} -1.0x10^{-2}	28.0±0.9	7.0x10^{-7}	Javanbakht *et al.* 2008
10	Metoprolol	Non-Covalent	2.0×10^{-7}-8.0×10^{-3}	55.4±1	1.26×10^{-7}	Tehrani *et al.* 2010
11	Chlormequat	Non-Covalent	6.2 x 10^{-6} – 1.0 x10^{-2}	53.2±0.5	4.1x10^{-6}	Kamel *et al.* 2008

Prof. Murray's group has utilized vinylated ligands for the complexation of cations Pb^{2+} [Zeng *et al.* 1997] & UO_2^{2+} [Murray *et al.* 1997] by polymerizing the complex in a rigid matrix of crosslinked imprinted polymer. After leaching, the imprinted particles were dispersed in PVC matrix and the sensing membranes were conditioned with the corresponding metal ions before potentiometric sensing studies. Our group has analogously designed and developed imprinted polymer inclusion membrane (IPIM) format based potentioselectrodes for Dy^{3+} [Prasad *et al.* 2006] and UO_2^{2+} [Metilda *et al.* 2007] ions which gave linear Nernstian slopes in the range $8 \times 10^{-6} – 1 \times 10^{-1}$M and 2.0×10^{-8} –1.0×10^{-2}M respectively. These membranes were casted by Moody and Thomas method [Craggs *et al.* 1974], using imprinted polymer materials prepared via trapping concept ie. by the immobilization of a non-vinylated ligand inside the polymer matrix, and dispersing the template removed polymer particles in polyvinyl chloride matrix. On changing the composition of internal electrolyte by incorporating Na_2CO_3 or complexing agents like EDTA, the transmembrane ionic fluxes from the internal electrolyte to the membranes were blocked, thereby lowering detection limits by 2 decades. Hutchins and Bachas [1995] reported a conducting polymer based nitrate selective electrode by electropolymerising pyrrole onto glassy carbon electrode in presence of $NaNO_3$. Calibration plots of near Nernstian slopes were observed (-56mV/decade over a linear range of 5×10^{-5}–0.5M Nitrate) with good selectivity coefficients over coexisting anionic species. The author has proposed an electrochemically mediated templating mechanism to explain the high selectivity.

1.7.3 Membrane potential changes by Organic guests

Potentiometric discrimination of organic guests based on membrane potential changes at membrane/aqueous solution interfaces can be due to the differences in both polar and nonpolar structures of the guests. Some of the molecules existing in the solution as cationic or anionic species can induce a potential flux due to the differential binding of analytes to the membrane surface. Membrane potential changes occurring on MIP membrane surfaces can be generally classified into i) Surface potential generated by charged molecules ii) Indirect sensing using indicator ions iii) Direct sensing of neutral molecules. Discrimination of polar structures or organic ion by selective changes in membrane potentials can be achieved on the basis of electrostatic interaction and/or hydrogen bonding with the polar moieties of guests. Such types of sensing of cationic guests like Cetrizine [Javanbakht *et al.* 2002], Melamine [Liang *et al.* 2009], Hydroxyzine [Javanbakht *et al.* 2008] Chlormequat [Kamel *et al.* 2008], Promethazine [Alizadeh and Akhoundian, 2010] , Metoprolol [Tehrani *et al.* 2010], Quinine [Kamel and Sayourb, 2009] etc has been reported using MIP based PVC membrane (IPIM) electrodes which has shown Nernstian responses. Wang *et al.* [2010] have reported surface molecular imprinted potentiometric sensors for some biomacromolecules like haemoglobin, myoglobin, cancer biomarkers and viruses which has widened the application potential of MIPs & Potentiometry. Recently Liang *et al.* [2010] reported a strategy based on indirect sensing of neutral molecule Chloropyrifos using an indicator anionic species which shows structural similarity towards the analyte Chloropyrifos. The response mechanism has been explained taking analogy of the dye displacement in optical sensing , instead here the indicator ion which is held less tightly in the cavities are dispossessed by the analyte having higher binding affinities, thereby creating surface potential. Odashima *et al.* [1998] reported anionic potentiometric responses to various phenolic guests by PVC liquid membranes incorporated with lipophilic macrocyclic polyamines under the pH conditions in which the phenols exists almost exclusively as their undissociated, neutral forms. On the basis of the results on potentiometric responses to neutral phenols, a model based on guest induced decrease in the amount of charge separated species at the membrane/aqueous solution interface was proposed. Zhou *et al.* [2004] reported anomalous positive response slope in the sensing of Methyl phosphonic acid anion using sol-gel imprinted polymer matrix on Indium tin oxide nanoscale transducers. Another innovative attempt to develop sensing MIP membrane prepared by in-situ polymerization was used for developing atrazine potentioselectrode [Agostino *et al.* 2006].

1.8 AIM OF THE THESIS

In view of the above summary on the history of MIP based potentioselectrodes, it has shown great future for the design and development of low cost field monitoring sensors. A proper integration of excellent molecular recognition properties of MIPs with potentiometric transduction can provide rugged sensors for organophosphorous environmental toxicants. In addition, there is scope for developing novel methods for synthesizing sensing surfaces using the remarkable versatility of MIPs. Hence first portion of the thesis deals with the design and develop MIP based potentioselectrodes in different formats for anionic and neutral organophosphorus compounds and study their sensing characteristics. Detailed studies of the role of various imprinting strategies, different membrane preparation protocols, effect of functional monomers, plasticizers etc and sensing parameters like sensitivity, selectivity, stability and reversibility of the developed potentioselectrodes has been demonstrated. Moreover the thesis envisages studies on the application potential of the developed sensors for environmental sample analysis.

Chapter 2

Anion Imprinted Methyl Phosphonic acid Sensor

2.1 Abstract

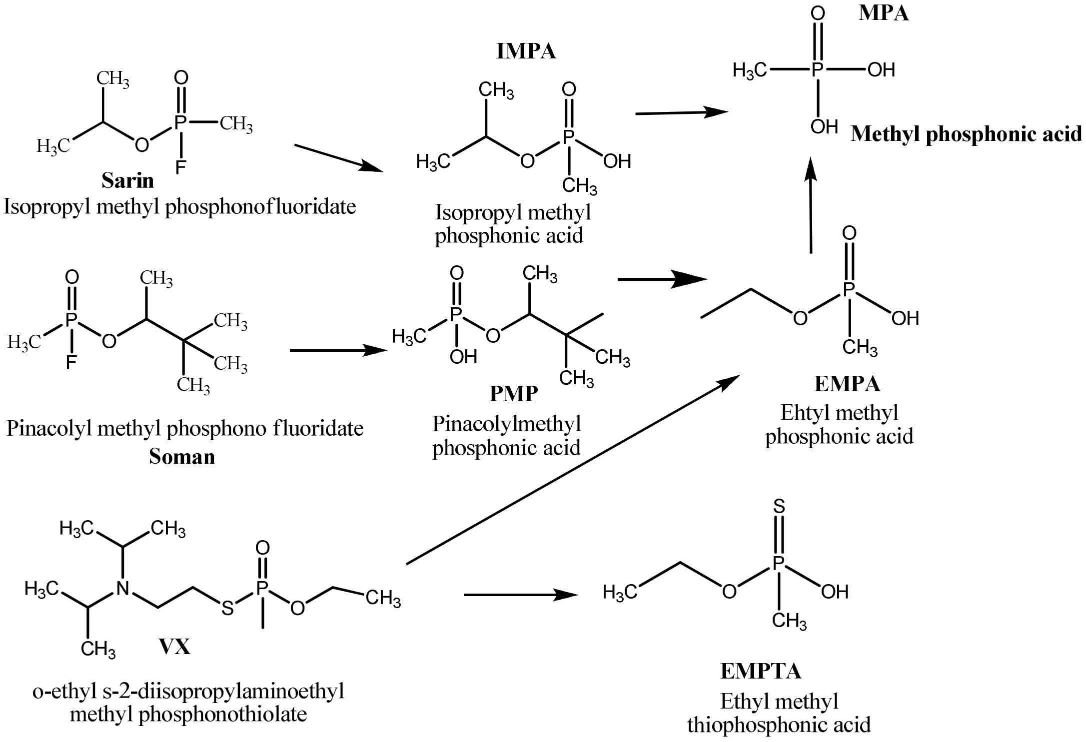
A biomimetic potentiometric sensor for the specific recognition of methyl phosphonic acid (MPA), the degradation product of nerve agent sarin, soman, VX etc was designed. Molecular imprinting of MPA has been conducted by Non-covalent strategy in presence of functional monomer methacrylic acid and ethylene glycol dimethacrylate as crosslinking monomer. The synthesized MIP materials were processed by grinding into microparticles and the template, methyl phosphonic acid was washed off by soxhlet extraction. Subsequently, sensing membrane was casted by dispersing the leached MIP particles in 2-nitrophenyloctyl ether (plasticizer) and embedded in polyvinyl chloride matrix. The sensor responds to MPA in the concentration range $1x10^{-6} - 10^{-1}$ *M with a detection limit of* $1x10^{-6}$ *M. The selectivity of the sensor has been tested with respect to chemical analogues such as phosphoric acid, sodium dihydrogen phosphate, organophosphorous pesticides and 2,4-D. The developed sensor has shown excellent reversibility and stability necessary for continuous environmental monitoring. The utility of the sensor was tested for field monitoring of MPA in spiked ground water samples.*

2.2 INTRODUCTION

Organophosphorous nerve agents (OPs) are a major issue worldwide, most visibly in the chemical weapons arena in relation to the decommissioning of arms stockpiles and the protection of personnel from warfare agents. As the nerve agents are hydrolyzed in the environment, the detection of degradation products such as methyl phosphonic acid (MPA) has been usually performed as the proof of the use of nerve agents [Liu and Lin, 2005; Tu, 1996]. Methyl phosphonic acid is the ultimate degradation product of organophosphorous nerve agents and pesticides as shown in the scheme 2.1 [Ashley *et al.* 1999]. There have been many innovations for the identification of MPA, including high performance liquid chromatography (HPLC), ion chromatography, HPLC–mass spectrometry and gas chromatography [Tu, 1987; Black *et al.* 1994]. Although these instruments do offer quantitative analysis, they are not optimal for rapid detection as they are beset with problems such as cost, requirement of sophisticated and often extensive analysis procedures and non-portability [Black *et al.* 1994; Mesilaakso, 1997; Nassar *et al.* 1999]. The methods that meet the real-time analysis such as fibre-optic sensors [Jenkins *et al.* 1999], surface acoustic wave devices [Nieuwenhuizen and Harteveld, 1997] or microbial biosensors [Mulchandani *et al.* 2001] often lack selectivity and do not have an optimal alarm ratio.

The development of highly selective chemical sensors for complex matrices of medical, environmental, and industrial interest has been the object of current research. Sensors of high sensitivity and selectivity still depend on biological recognition systems. But the inadequate stability and reversibility of field-tested biosensors prevent long-term monitoring and applications in harsh conditions. These demands can be met with robust MIP coated chemical sensors [Janata and Josowicz, 1998]. The advantages of sensors based on imprinted polymers are their high stability, which allows long operation under harsh conditions without loss of sensitivity, and their high specificity, which are comparable to that of polyclonal antibodies.

Molecular recognition at interfaces not only has relevance to biological systems but is also important for modern applications such as high sensitivity sensors. Selective binding of guest molecules in solution to host molecules located at solid surfaces is crucial for optoelectronic and electrochemical detection of analyte substances.



Scheme 2.1 *Degradation pathways of organophosphorous nerve agents*

Electrochemical molecular recognition is an expanding research area at the interface of electrochemistry and supramolecular chemistry [Beer *et al.* 1999; Boulas *et al.* 1998]. Three strategies have been applied to the electrochemical detection of the formation of receptor-anion complexes: 1) extraction of a charged guest into a membrane by a non electroactive host and detection of the resulting membrane potential (ion-selective electrodes (ISEs), chemically modified field-effect transistors (CHEMFETs), potentiometric sensors, 2) detection of a current/potential perturbation of the properties of a redox-active host on complex formation (voltammetric/amperometric sensors) and 3) production of a chemically modified electrode (CME) consisting of a redox-active matrix and an anion-selective binding site.

In view of the virtues of potentioselectrodes over spectral and other electrochemical approaches as described in chapter 1, we have selected potentiometric transducers to integrate with the tailor-made molecular recognition entities viz. MIPs for the selective sensing of methyl phosphonic acid (MPA). Methyl phosphonic acid exists as univalent anion under alkaline conditions. The design of anion receptors is particularly challenging due to various reasons. Anions are larger than isoelectronic cations and therefore have a lower charge to radius ratio [Beer and Gale, 2001; Shannon, 1976]. This means that electrostatic binding interactions are less effective for anions when compared to smaller cation. Additionally anions may be sensitive to pH values (becoming protonated at low pH and so losing their negative charge), thus receptors must function within the pH window of their target anion. Anionic species have a wide range of geometries and therefore a higher degree of design may be required to make receptors complementary to their anionic guest.

Many of the neutral lipophilic urea- and thiourea-containing receptors have been successfully incorporated into ion-selective electrodes (ISEs) which are capable of selective anion detection. For example, Umezawa and co-workers have reported chloride-selective membranes based on bis-urea systems [Xiao *et al.* 1997]. Reinhoudt and co-workers have incorporated an array of lipophilic uranyl salophane derivatives into chemically modified field-effect transistor (CHEMFET) membranes and shown that these devices are capable of selectively detecting a range of anions (for example, fluoride may be detected in the presence of a 150- fold excess of SCN^-), and is dependent upon the lipophilic and hydrogen-bond donor/acceptor substituents near the uranyl binding site of the receptor [Antonisse *et al.* 1998a; 1998b; 1999].

Mosbach, one of the pioneers of molecular imprinted polymers and his coworkers has reported the first MIP based ion selective electrode for Ca (II) and Mg (II) ions [Rosatzin *et al.* 1991]. Murray et al reported potentiometric transducer based imprinted polymer inclusion membrane (eventhough not named by authors) sensors for uranium [Murray *et al.* 1997] and lead [Zeng and Murray, 1996], where in the MIP particles are dispersed in polyvinyl chloride matrix. From the previous literature reports, it is clear that very few reports are there on MIP based anion sensing potentiometric sensors [Ewen and Steinke, 2007]. As there is growing threats from various environmentally toxic anions and in view of the remarkable selectivity offered by MIPs coupled with the simplicity of potentiometric sensors, the development of MIP based potentiometric sensors can provide portable, cost effective and rugged chemical sensors. Bachas *et al.* has reported a conducting polymer based nitrate selective electrode by electropolymerizing pyrrole onto glassy carbon electrode in presence of $NaNO_3$ [Hutchins and Bachas, 1995]. Calibration plots of near Nernstian slopes were observed (-56mV/decade over a linear range of $5x10^{-5}$ – 0.5M Nitrate) with good selectivity coefficients over coexisting anionic species. The authors have proposed an electrochemically mediated templating mechanism to explain the high selectivity. Zhou *et al.* [2004] reported MPA anion sensing potentioselectrode for the first time by employing surface imprinting technique coupled with a nanoscale transducer, indium tin oxide. The sensing membrane was prepared by the co-adsorption of the template methyl phosphonic acid (MPA) and octadecyl trichloro silane thereby forming a monolayer on indium tin oxide substrate. A Positive potential change has been reported with a slope of 30mV per decade in lower concentration range. Again, after MPA molecules were inserted into the ODS layer without dissociation, ITO could still translate a molecular recognition process into potential alteration. But the imprinting is done in the absence of any cross linking agent which may be responsible for the low specific molecular recognition property of MIPs. In the present work we have synthesized cross linked acrylic polymer for the selective potentiometric sensing of MPA. Non-covalent imprinting strategy has been adopted for synthesizing the MPA imprinted MIP particles. During this study we have varied the monomer combination for getting optimum potential response during the rebinding of the analyte. The findings pertaining to the potentiometric sensing characteristics and influence of various critical parameters are described in this chapter.

2.3 CONSTRUCTION OF THE MPA SENSOR

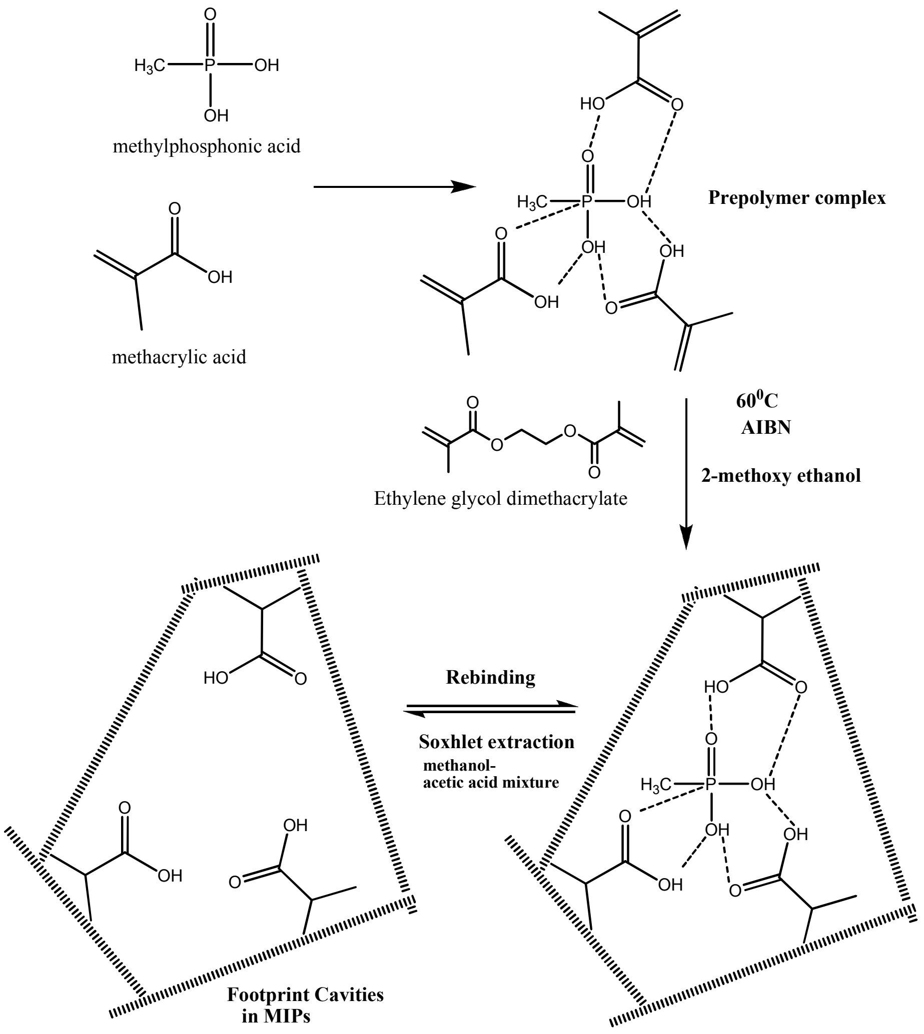
The fabrication of MPA imprinted material based electrode involves 2 steps viz., (i) synthesis of MPA imprinted polymer material and (ii) preparation of membrane by dispersing MPA imprinted polymer particles in PVC matrix.

2.3.1 Preparation of MPA imprinted and non-imprinted polymer particles

The synthesis procedure for preparing MPA imprinted polymer particles were shown in Scheme 2.2. MPA imprinted polymer particles were prepared by taking 1mmol of MPA and 8 mmol of methacrylic acid (MAA) in 50 ml round bottom flask and the mixture was allowed for prearrangement for about 5 min. Subsequently, ethylene glycol dimethacrylate (EGDMA) (32 mmol), Azo-bis isobutyronitrile (AIBN) (2 mmol) and 10 ml of 2-methoxyethanol were added. The mixture was purged with N_2 for 5 min and the flask was sealed under this atmosphere. It was then kept for stirring in an oil bath maintained at 65^0C to start polymerization process. After 1 hr, the obtained molecular imprinted polymer (MIP) materials were ground and sieved, and the particles with sizes between 50 and 105 μm were collected. MPA and unpolymerized monomers were removed by soxhlet extraction with 100 ml of 1:1 methanol–acetic acid by refluxing for 12 hr. Then, the particles were again suspended in acetone and allowed to settle for 4 hr. The sedimented particles were discarded and those not sedimented were collected by centrifugation. The particles collected were suspended in acetone again and allowed to settle for 4 hr, followed by centrifugation. The resulting MIP particles were dried to constant weight under vacuum at 60°C and were used in the following experiments. Non-imprinted polymer (NIP) particles were prepared analogously without the addition of MPA during polymer material preparation.

2.3.2 Preparation of sensing membrane

The polyvinylchloride (PVC) membrane sensors were fabricated by following the general procedure mentioned below. MPA imprinted or non imprinted polymer particles (90 mg) were dispersed in 0.2 ml of 2-nitrophenyl octyl ether or Dioctyl phthalate (DOP) or Bis-2 ethyl hexyl sebacate (BEHS) or Tris-2 ethyl hexyl phosphate (TEHP) and were added to 2.5 ml of tetrahydrofuran (THF) containing 90 mg of PVC. The resulting solution was homogenized in a sonicator and then poured in a Teflon mould of 21mm of internal diameter. THF was allowed to evaporate at room temperature. The polymer membranes thus obtained have a thickness of ~0.45 mm. The membranes were glued to one end of a pyrex glass tube with Araldite. The tube was then filled with an internal filling solution of 10^{-3}M of MPA. The sensor was kept in air when not in use.



Scheme 2.2 *Preparation of MPA imprinted polymer with Footprint Cavities*

2.4 OPTIMIZATION STUDIES

2.4. 1 Influence of functional monomer

The influence of functional monomer on the response characteristics of MIP-based potentiometric sensor for MPA was first investigated. In these studies, two such functional monomers, viz. MAA or 4-vinyl pyridine (VP) were used for preparation of MIP particles as mentioned in Section 2.3.1, under exactly identical conditions except

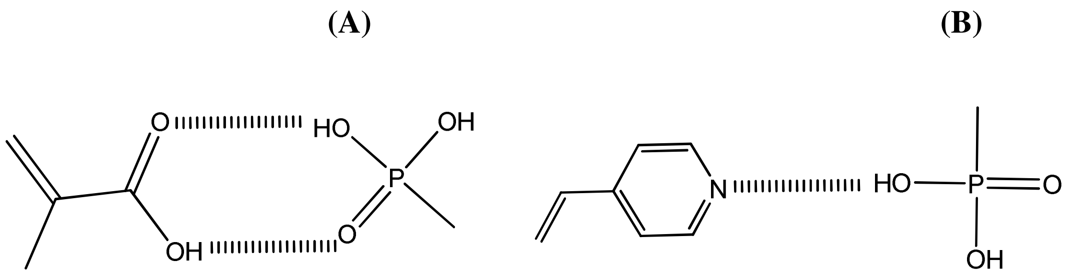
MAA is replaced by same concentration of 4-vinyl pyridine. Sensors were fabricated using MAA- or VP-based MIP and corresponding NIP particles and response studies were conducted for MPA as per the procedure described in Section 2.10.2.

Table 2.1 *Response study of MPA sensors fabricated using MAA and 4-vinyl pyridine as functional monomers*

[MPA], M	Potential response (mV/decade)			
	NIP[a]	MIP[a]	NIP[b]	MIP[b]
10^{-6} to 10^{-5}	-	+2	-	+3
10^{-5} to 10^{-4}	-	+2	-	+8
10^{-4} to 10^{-3}	+2	+5	+9	+17
10^{-3} to 10^{-2}	+35	+43	+48	+59
10^{-2} to 10^{-1}	+36	+45	+49	+59

[a] 4-Vinyl pyridine [b] MAA

The results obtained are shown in Table 2. 1, from which it is clear that in all decade change of concentration, the response is higher for the sensor fabricated using MIP particles prepared from MAA as functional monomer compared to VP. This observation can be explained by the stronger H-bond interaction of MPA anion with MAA compared to VP. Moreover molecular imprinting requires multiple short-range weak interactions that act over short distances. The efficiency of imprinting increases as the number of interacting sites between functional monomer and the template increases. In the present case there are two interacting sites in the case of MAA whereas only one site is available for VP as shown in scheme 2.3. However, when comparing MIP with NIP particle-based sensors using MAA or VP as functional monomer showed imprinting effect in the entire concentration range. Hence, in all subsequent studies of MPA sensor, MIP particles prepared using MAA as functional monomer is preferred.



Scheme 2.3 *Binding interactions between functional monomers and template (A) MAA- MPA (B) VP-MPA*

2.4.2. Effect of membrane composition

Literature reports on ionophore based conventional potentiometric sensors show that the response behaviour of the sensor depends on various features of membranes such as the properties of the plasticizer and, nature and amount of ion recognizing materials [Moody *et al.* 1988; Hassan *et al.* 2001; Gupta *et al.* 1999]. Thus, different aspects of the membrane preparation using MPA imprinted polymer particles were optimized on similar lines.

2.4.2. 1 Nature of plasticizer

One of the primary requirements of polymers employed in ISE membranes is that their glass transition temperature (Tg) should be less than room temperature [Fiedler and Ruzicka, 1973]. When this requirement is met, the membranes are sufficiently fluid under ambient conditions to allow reasonable ionic conductivities, diffusion of membrane components, and have mechanical properties amenable to routine processing and handling [Bakker *et al.* 1997; Armstrong and Horvai, 1990]. However, most polymers employed do not meet this physical requirement (e.g., Tg for

PVC is 80 °C) and must be plasticized. For PVC-based ISEs, typical weight ratios of polymer to plasticizer are approximately 1:2 [Craggs *et al.* 1974; Moody *et al.* 1970]. In such a highly plasticized state, the membrane is essentially an extremely viscous liquid. Additionally, since the membranes comprise a high percentage of plasticizer, this component acts as solvent for all membrane constituents and, hence, should be compatible with these species. Aside from yielding desired physical properties of the membranes and acting as a solvent, studies have demonstrated that the nature of the plasticizer can have an effect on the performance of the ISE.

Addition of appropriate plasticizer leads to optimum physical properties and ensures high mobility of MPA ions in the membrane. These solvent mediators strongly influence the working concentration range of potentiometric sensors. The effect of different plasticizers on the performance of MPA sensor was investigated. Fig.2.1 shows the potential response of MPA sensor with different plasticizers NPOE, BEHS, TEHP and DOP. Of these, the membrane with NPOE offered higher sensitivity with a Nernstian response of 59.0 mV over the range 1×10^{-3} to 1×10^{-1} M and lower detection limit of 1×10^{-6} M. This may be attributed to the higher dielectric constant of 24.0 for NPOE when compared to that of DOP ($\in = 5.0$), BEHS ($\in = 4.0$) and TEHP ($\in = 4.8$) as seen from Table 2.2. The dielectric constant of the plasticizer plays a major role in the diffusion process. In high dielectric constant media, ion-pairs can dissociate more readily. The individual ions have a higher diffusion coefficient than a neutral and bulky ion-pair consisting of the target solute and the carrier [Nghiem *et al.* 2006].

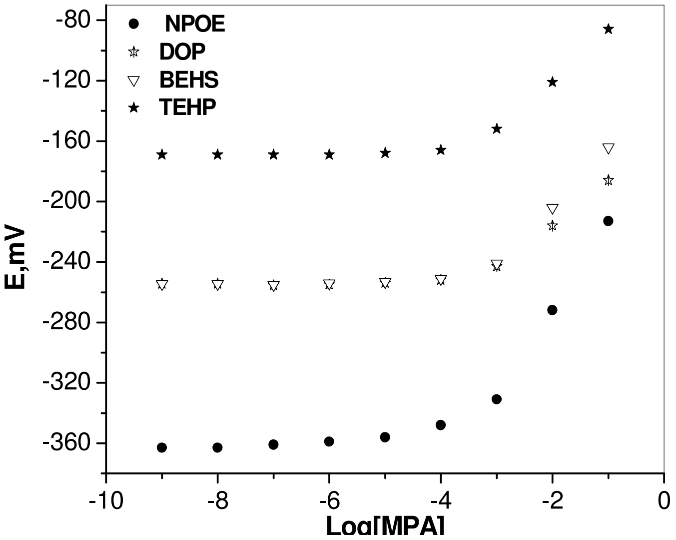


Figure 2.1 *Potential responses of MPA potentiometric sensor with different plasticizers*

This observation is in tune with earlier report on conventional thallium (I)-selective PVC membrane electrode employing tetrathia macrocycle as ionophore [Singh and Saxena, 2005]. Shamsipur and coworkers have compared conventional lanthanum (III) PVC membrane electrodes constructed with three different plasticizers and found that the sensitivity can be directly related to dielectric constant of the plasticizer [Shamsipur *et al.* 2002]. Thus, of the three plasticizers studied by above authors, acetophenone (∈ = 17.4) gave better sensitivity compared to dibutylphthalate (∈ = 8.5) and benzyl acetate (∈ = 5.1). In a similar vein, MPA sensor gave narrow linear response range and poor detection limits with plasticizers like BEHS, TEHP and DOP compared to NPOE. It is pertinent to mention here that MPA membranes were found to be brittle in the absence of plasticizer and cannot be used for recording sensor performance.

Table: 2.2 *Potential response of MPA sensor prepared using different plasticizers*

[MPA], M	Potential response (mV/decade)			
	NPOE (ε =24.0)	DOP (ε =5.0)	BEHS (ε =4.0)	TEHP (ε =4.8)
10^{-6} to 10^{-5}	+3	+0.9	+0.5	+0.5
10^{-5} to 10^{-4}	+8	+1.5	+2	+2.7
10^{-4} to 10^{-3}	+17	+9	+10.5	+13.0
10^{-3} to 10^{-2}	+59	+27	+37	+31

2.4.2.2. Effect of MIP particles to PVC ratio

The ratio of ionophore to PVC influences the working concentration range, slope and response time in case of conventional ionophore-based sensors. This was evident from the accounts of several researchers in the past, for e.g., thallium(I) sensor reported by Singh and Saxena [2005], yetterbium(III) and lanthanum(III) sensors reported by Ganjali and coworkers [Ganjali *et al.* 2003a; 2003b], etc. In case of imprinted polymer ion selective electrodes, viz. dysprosium (III) and atrazine [Prasad *et al.* 2006; 2007], we observed that the ratio of PVC to imprinted polymer particles was found to play a key role in the efficiency of sensors since the amount of imprinted polymer particles determines the number of binding sites available for recognition. Hence, a similar study was conducted with MPA sensor and the results obtained are shown in Table 2.3 from which it is clear that the membrane having MIP particles to PVC ratio of 1:1 gave the best response. In case of membrane with the ratio 0.5:1, the total number of binding sites available for MPA is relatively lower for the membrane to respond effectively. On the other hand, during the preparation of membrane with the ratio 2:1, the MPA imprinted polymer particles are dispersed non-uniformly causing poor response behavior.

Table.2.3 *Effect of weight ratio of MIP particles to PVC on MPA sensor response*

Weight of MIP particles (mg)	Weight of PVC (mg)	Weight ratio	Working concentration range (M)	Response slope (mV/ decade)
45	90	0.5:1	10^{-4} - 10^{-2}	+24.8
90	90	1:1	10^{-3}- 10^{-1}& 10^{-6} – 10^{-3}	+59.0 &+9.2
180	90	2:1	10^{-4} - 10^{-2}	+14.3

2.4.3 Effect of pH

The effect of pH of test solution on the performance of MPA sensor was studied by varying the pH in the range 9.0–11.0 in steps of 0.2 after addition of 5ml of 1.0 M Tris–(hydroxymethyl)aminomethane (Tris) buffer. The results show that the optimal pH for constant and maximum response characteristics over the entire concentration range of MPA (1×10^{-6} to 10^{-1} M) is 9.8–10.2 and an optimum pH of 10 was selected for further studies.

2.5 RESPONSE TIME AND DYNAMIC RESPONSE STUDY

Response time is one of the important criteria of a suitable sensor. In the present case it was found that about 80% of the response was obtained within 5 mins while it took almost 10 mins for the equilibrium response to occur. This may be due to the slow binding of MPA anion at the membrane interface due to the low charge density. Dynamic response study is yet another factor that measures the reversibility of the sensor. An ideal sensor should not exhibit any "memory effect" even if it is exposed to widely varying concentrations of analyte.

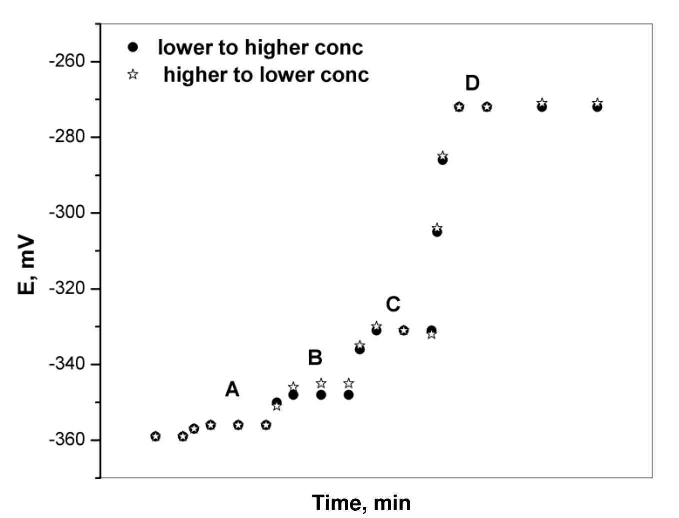


Figure 2.2 *Dynamic response study of the MIP based MPA potentiometric sensor for stepwise concentration change of MPA A: 1x10^{-6} M, B: 1x10^{-5} M, C: 1x10^{-4} M, D: 1x10^{-3} M.*

This implies that same response should be reproduced in a particular concentration range whether concentrations are varied in ascending or descending order ensuring its utility for continuous monitoring. The response time was recorded by changing the MPA concentration in test solution over a concentration range of 1×10^{-6} to 1×10^{-2}M (see Fig. 2.2). To evaluate the reversibility of MPA sensor, a similar procedure in opposite direction was studied. The measurements performed in the sequence of high to low concentration (from 1×10^{-2} to 1×10^{-6} M) indicate that the MPA sensor was reversible analogous to conventional ionophore based sensors.

2.6. SENSITIVITY AND PRECISION

The potential responses of MPA imprinted (MIP) and non imprinted (NIP) polymer membrane sensors fabricated under optimal conditions, as obtained from the above studies, were checked and the results obtained are shown in Fig. 2.3a.

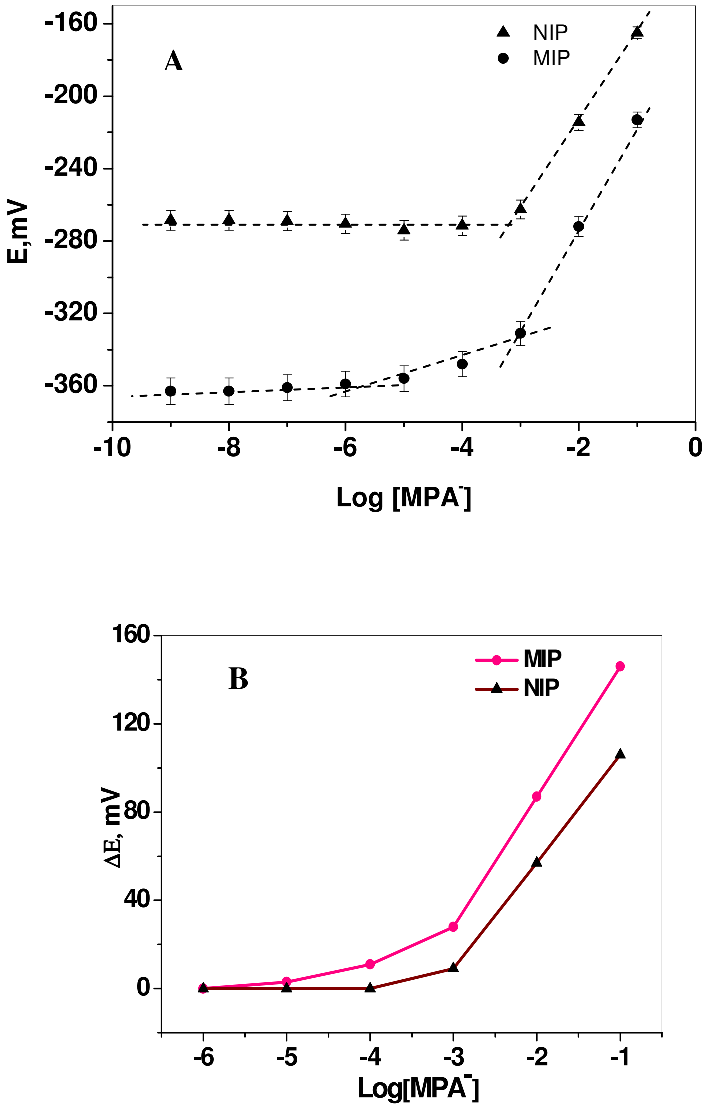


Fig2.3 *a) Potential responses of potentiometric sensors with NIP and MIP membranes and b) represents corresponding calibration graph for MIP & NIP*

As seen from the figure, the plot obtained for MPA imprinted polymer sensor (based on duplicate measurements at each concentration) offers linear response in the ranges 10^{-6} to 1×10^{-3}M and 1×10^{-3} to 1×10^{-1} M with slopes of +9.2±0.1 and +59.0±0.5mV/decade (See Fig 2.3b). The limit of detection was calculated to be 1×10^{-6}M based on IUPAC definition [Buck and Lindener, 1994]. On the other hand, the non-imprinted polymer based membranes do not respond to MPA below 1×10^{-4}M and gave linear response for MPA in the concentration range 10^{-3} to 10^{-1} M only. Better response characteristics of MPA imprinted sensor over non-imprinted polymer-based sensor in the entire concentration range is attributed to significant imprinting effect. In the present study we have obtained an anomalous positive potential change unlike expected negative potential change for anions. Also non-Nernstian potential change was observed in the lower ranges while Nernstian response was obtained in the higher ranges of 10^{-3} to 10^{-1} M. The comparatively sub Nernstian responses at lower ranges may be attributed due to the fact that the rebinding process is slow in aqueous media.

2.7. SELECTIVITY STUDIES

The potentiometric output of the MIP-based MPA sensor was studied with some common organophosphorous pesticides, herbicides and chemicals like H_3PO_4 and NaH_2PO_4 that are most likely to interfere. The results obtained for change of concentration from 10^{-6} to 10^{-4}M are shown in Fig.2.4. MIP based potentioselectrode has shown superior selectivity for methyl phosphonic acid when compared to coexisting analytes. The ratio of potential change corresponding to the analyte with respect to that of interferents defined as the response factor is an indicator of the selectivity of the analyte. The response factor given in Table 2.4 exemplifies that the MIP based membrane has shown several orders of response compared to the coexisting interferents. Response factor is defined as the ratio of potential change obtained with the developed sensor for the analyte MPA with respect to that obtained for interferents. It can quantify how many folds the developed sensor is selective to the analyte under consideration than the coexisting interferents.

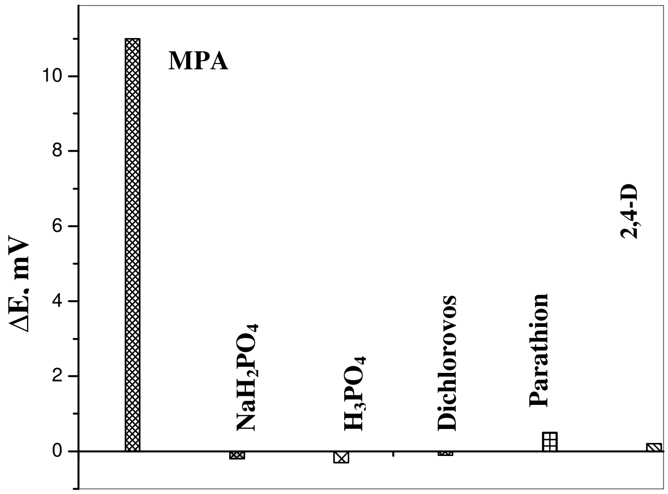


Figure 2.4 *Potential change of MPA sensor to MPA and interferences at 1x10^{-4} M concentrations*

Table 2.4 *Response factors of MPA over other interferents*

S.L.No	Interferents	Response factor of MPA over interferents
1	NaH_2PO_4	220
2	H_3PO_4	330
3	Dichlorovos	110
4	Parathion	22
5	2,4-D	55

2.8. STABILITY AND REUSABILITY

The important criteria required for any sensing device in addition to sensitivity and selectivity is stability and reusability. The above developed MPA sensor was found to be stable for 2 months and can be reused for more than 20 times without any loss in sensing ability.

2.9. ANALYTICAL APPLICATIONS

Since organophosphorus pesticides, H_3PO_4 and NaH_2PO_4 did not show false positive signals, it was decided to analyze natural water samples spiked with known amounts of MPA. The results obtained are shown in Table 2.5 from which it is clear that MIP based MPA sensor can reliably be used for monitoring MPA in natural waters contaminated with chosen organophosphorus pesticides or H_3PO_4 or NaH_2PO_4.

Table 2.5 *Analysis of natural water (45 ml of sample + 5 ml of pH 10.0 Tris buffer)*

Sample	Interferent, 100 µM	Concentration of MPA, µM	
		Added	Found*
Natural water	-	-	-
"	-	100	99 ±1.0
"	Dichlorovos + Malathion	100	98 ±1.0
"	$H_3PO_4 + NaH_2PO_4$	100	98 ±1.0
"	Dichlorovos + Malthion + $H_3PO_4 + NaH_2PO_4$	100	99 ±1.5

* Average of 3 successive determinations

2.10: EXPERIMENTAL

2.10.1. Reagents

Methyl phosphonic acid (MPA), Dichlorovos, Parathion and 2, 4-D were obtained from SUPELCO, USA. Methacrylic acid (MAA), 4- Vinyl pyridine (VP), Ethylene glycol dimethacrylate (EGDMA), 2, 2'-Azobisisobutyronitrile (AIBN), 2-nitrophenyloctylether (NPOE), di-n-octylphthalate (DOP), bis(2-ethylhexyl) sebacate (BEHS), tris(2-ethylhexyl) phosphate (TEHP), and high molecular mass Poly(vinyl chloride) (PVC) were purchased from Aldrich (Milwauke, USA). All other chemicals including NaH_2PO_4 and H_3PO_4 were of analytical reagent grade obtained from E-Merck, India. Deionized water was used throughout the studies.

2.10.2. Potentiometric response studies

Stock standard solution of (0.1 M) MPA was prepared by dissolving 0.4801 g of MPA in 50 ml of deionized water. The solutions of 1×10^{-6} to 1.0×10^{-2}M of MPA were prepared by serial dilution of the stock solution. The sensor was conditioned in 25 ml of 0.1M Tris buffer with pH 10 for 30 min. The response of the sensor was examined by measuring the electromotive force (EMF) of the following electrochemical cell. Ag, AgCl 1.0× 10^{-3}M MPA| PVC membrane | sample solution || KCl (saturated)|Hg_2Cl_2, Hg. The potential response of the sample solution containing varying amounts of MPA in 50ml of 0.1MTris buffer (pH 10) was measured. The EMF was plotted as a function of MPA concentration.

2.10.3. Analysis of ground water samples

Ground water samples (after adjusting the pH to 10 in presence of 0.1M Tris buffer with HCl or NaOH), spiked with known amounts of MPA were analyzed by following the analytical procedure as described in Section 2.10.2 using the above developed MPA imprinted potentiometric sensor.

2.11. CONCLUSIONS

We have described a simple and elegant way of designing potentiometric sensor for methylphosphonic acid based on molecular imprinting concept. The sensor has shown improved selectivity over structurally or chemically related compounds. The developed MIP sensor responds to MPA with linear responses of + 9.2± 0.1 mV and + 59 ± 1mV/decade in the ranges 10^{-6} -10^{-3} and 10^{-3} to 10^{-1}M. Optimum membrane composition and functional monomers for effective sensing characteristics were identified. The notable feature of the present sensor was the anomalous positive slope for the analyte existing as anionic species under the experimental conditions. The stability, reusability, portability and absence of memory effect enable the present sensor device to find ready use in field monitoring studies for MPA as demonstrated by the ground water analysis.

Chapter 3

Molecule Sensing Potentioselectrode For Phorate

3.1 Abstract

A biomimetic potentiometric sensing electrode was developed for the trace determination of phorate (O,O-diethyl S-ethyl thiomethyl phosphorodithioate) in natural waters. Phorate imprinted polymer particles were synthesized by precipitation polymerization method using methacrylic acid as functional monomer and the template was washed off by soxhlet extraction. The sensing element was fabricated by the inclusion of phorate imprinted polymer materials in the polyvinyl chloride (PVC) matrix. The sensor surface can be reused without conditioning unlike most other conventional sensors. Operational parameters such as amount and nature of plasticizers and sensing material, pH and response time were optimized. The response performance of the non-imprinted (NIPIM) and imprinted polymer inclusion membrane (IPIM) sensors for phorate were compared under optimum conditions. The IPIM sensor responds to phorate in the concentration range $1x10^{-9}$ *to* $1x10^{-5}$ *M with a detection limit of* $5x10^{-9}$*M. The selectivity was tested with various common organophosphorus (OP) pesticides and herbicides. In addition to superior sensitivity and selectivity of IPIM over NIPIM-based sensor, IPIM-based phorate sensor was found to be stable for 3 months and can be used for more than 40 times without any loss in sensitivity. The applicability for analyzing ground, river and tap-water samples was successfully demonstrated via recovery studies.*

3.2 INTRODUCTION

Advances in “host-guest chemistry” for the design and synthesis of artificial molecules that mimic bioreceptor functions have stimulated a number of research groups to develop novel types of sensors where synthetic receptors are used as sensory elements [Odashima *et al.* 1991; 1994]. Host-guest interactions, occurring either in solutions or at membrane surfaces, is a fundamental chemical process that controls many important biological reactions such as antigen-antibody reactions, enzymatic reactions, carrier or channel mediated transport and receptor reactions involving signal transduction. There are two representative modes of signal transduction that could be induced by host-guest complexation at surfaces ie. membrane potential change and membrane permeability change [Sugawara *et al.* 1989]. Of these, the guest induced membrane potential change is the principle of signal transduction that has been extensively exploited for potentioselectrodes. Many electrodes with excellent selectivities for inorganic cations have been developed by using totally synthetic hosts such as crown compounds and acyclic ligands [Buck *et al.* 1990]. On the otherhand, there have been only few examples for electrodes that strongly respond to discriminate between organic guests. The main principles employed so far for these electrodes are the potentiometric discrimination based on lipophilicity or chirality of organic guests [Amman *et al.* 1983]. There are 3 new principles for discrimination based on interaction of organic guests by the membrane potential changes induced by host-guest complexation at the membrane surface. The first two principles are the potentiometric discrimination based on interaction with specific functional groups of organic guests, such as charged groups or hydrogen bonding groups. The third principle is the potentiometric discrimination based on the recognition of the steric shapes or non-polar moieties of organic guests by inclusion in a cavity of host having a well defined structure.

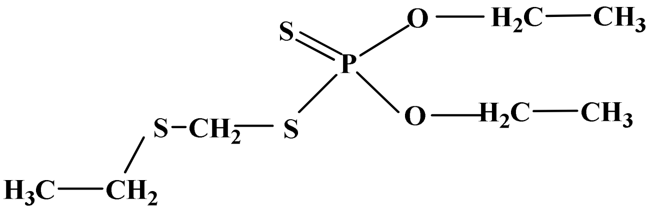
Molecular recognition of electroinactive species affecting the response of ISEs can be used for broadening their application. This area of electrochemical sensor development widely utilizes selective binding of electroneutral species, affecting either reactions on the electrode surface (redox-reactions, enzyme catalyzed reactions, etc.) or electrical parameters of the electrode/sample interface (usually resistance and capacitance). Biological molecular recognition involving signal transduction, occurring either in a homogeneous solution or at a membrane/aqueous solution interface, frequently plays a significant role in such functions as cellular signaling, neurotransmission, hormone function, protein transport, genetic regulation, etc. The development of new molecules with a function of molecular recognition involving signal transduction is a challenging problem from the fundamental aspects of designing and synthesizing molecules with tailored functions as well as their application to new types of materials or reagents including those for analytical use [Atwood *et al.* 1996; Gokel, 1997]. The development of "potentiometric" sensors for neutral molecules is intrinsically a puzzling problem but involves a significant question concerning how uncharged species can influence charge separation across the membrane interface and induce a potentiometric response [Ito *et al.* 1998].

Guest-induced changes in membrane potentials are one of the representative modes of electrochemical signal transduction by molecular recognition at the interface of an organic membrane and an aqueous solution. Potentiometric sensing of neutral molecules is still an open challenge for analytical chemists. It is also possible that molecular recognition on the electrode surface leads to the decrease in the binding activity of an enzyme or an antigen by blocking its binding sites, which is detected as a decrease of an indicator reaction signal. These principles are widely utilized in biosensors and in ion-channel mimetic sensors with artificial molecular receptors [Sugawara *et al.* 1987; 1989]. The main difficulty in preparation of these kinds of chemically modified electrodes is the necessity to avoid formation of pinholes in the recognition or support layer to prevent shorting of electrode.

Odashima has reviewed the trends in molecular recognition involving membrane potential changes induced by host-guest complexation with inorganic and organic guests [Odashima, 1998]. The review elaborates membrane potential changes created due to inorganic guests, discrimination of organic guests based on polar & non polar structures and the membrane potential changes induced by neutral molecules. Umezawa and coworkers have reported anionic potentiometric responses to various phenolic guests by PVC liquid membranes incorporated with lipophilic macrocyclic polyamines under the pH conditions in which the phenols exist exclusively as their undissociated, neutral forms [Ito *et al.* 1998].

Molecular imprinted polymers are unique molecular recognition scaffolds mimicking the spatial and size specific recognition ability of enzymes and antigen-antibody interactions. Hitherto there are no reports on the MIP based potentiometric sensing of neutral molecules by direct methods. Recently Qin and coworkers reported a strategy based on indirect sensing of Chloropyrifos using an indicator ion which shows structural similarity towards the analyte viz Chloropyrifos exists as anionic species at neutral pHs unlike the analyte [Liang *et al.* 2010]. The sensing occurs by indirect means taking analogy of the dye displacement of optical sensors, instead here the indicator ions which are held less tightly in the cavities are dispossessed by the analyte of higher binding affinities & thereby creating surface potential.

Natural waters are contaminated with various pesticides and insecticides because of their widespread use in commercial and residential applications. We have selected a widely used organophosphorous pesticide, Phorate as our target analyte which exists as neutral undissociated molecule under the experimental conditions. These biomimetic receptors can readily and unequivocally distinguish the target OP pesticide from structurally and functionally similar class of compounds. Phorate (O, O-diethyl S-ethyl thiomethyl phosphorodithioate, CAS No: 298-02-2) Scheme 3.1, is a widely used, typical thioether containing organophosphate pesticide. The U.S Environmental Protection Agency market estimates based on crop life America annual surveys for 2001 usage include 2-3 million pounds of active ingredient of phorate in United States alone [Mahajan *et al.* 2006]. Rao and coworkers has done extensive survey on biomimetic sensors and an overview of biomimetic sensors for toxic pesticides and inorganics based on various transducers has been reported [Rao *et al.* 2007]



Scheme 3.1 *Chemical Structure of Phorate*

As of now, there are no literature reports on the utilization of imprinted polymer inclusion membrane (IPIM) based potentiometric sensors for the trace determination of

OP pesticides in the neutral form. In this chapter we have made use of the effective molecular recognition property of MIPs and the subsequent potential gradient generated upon template binding.

3.3 CONSTRUCTION OF PHORATE SENSOR

The sensing membrane was fabricated by the sequential approach as described in section 2.3. It consists of two steps viz. (i) Synthesis of phorate imprinted and non-imprinted polymer materials (ii) preparation of sensor by dispersing the MIP particles in PVC matrix.

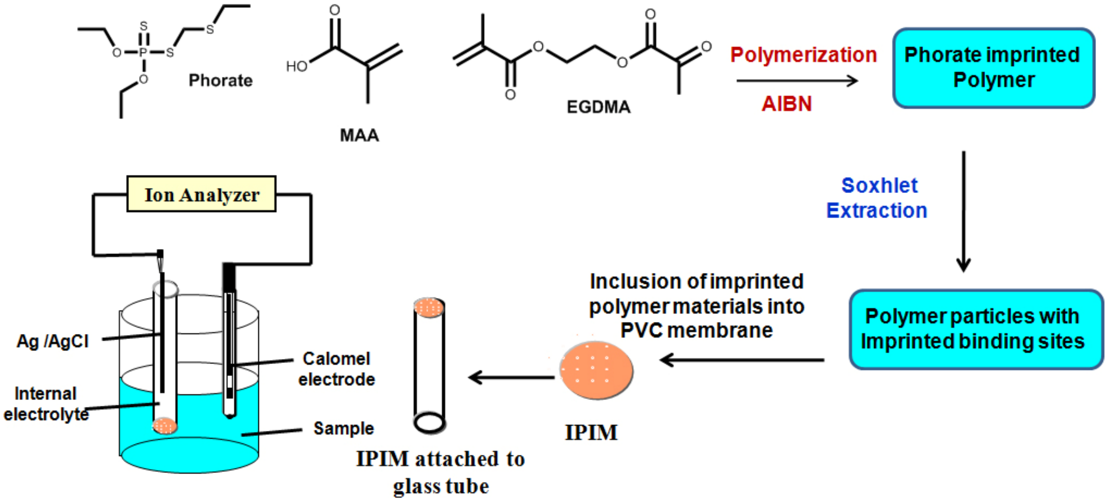
3.3.1 Preparation of phorate imprinted and Non-imprinted polymer materials

Phorate imprinted polymer materials were prepared by precipitation polymerization method. 1 mmol of phorate and 8 mmol of MAA were taken in an RB flask and allowed for prearrangement for 5 min. It is then followed by the addition of 20 mmol of EGDMA and 2 mmol of AIBN and 20 mL of porogen (Toluene). The mixture was then purged with N_2 for 5 min and the flask was sealed under inert atmosphere. It was then kept stirring in an oil bath at 60°C to start the polymerization process. After 24 hrs, the obtained polymer materials were crushed and sieved and the particles in the size range 50-105 μm were collected. The polymer materials were washed with acetone to remove the unpolymerised reactants. The phorate template was removed by soxhlet extraction with glacial acetic acid for 1 hr followed by toluene for 2 hr. Then, the particles were suspended in acetone and allowed to settle for 4 hr. The sedimented particles were discarded and those not sedimented were collected by centrifugation. This process was repeated two times. The resulting MIP particles were dried to constant weight under vacuum at 60°C and used in the following experiments.

Non-imprinted polymer (NIP) particles were prepared analogously without the addition of phorate during polymerization.

3.3.2 Membrane preparation and Sensor fabrication

The IPIM and corresponding non-imprinted (NIPIM) sensor was constructed by following the general procedure mentioned below. The phorate imprinted and non-imprinted polymer particles synthesized in section 3. 3.1 (90 mg) were added to 0.2 mL of NPOE [DOP, BEHS, TEHP] and 2.5 mL of THF containing 90 mg of PVC. The resulting solution was homogenized and poured into a Teflon mould of 17 mm diameter. Polymer membrane of ~ 0.45 mm thickness was obtained by the slow evaporation of the solvent.



Scheme 3.2 *Sequence of preparing phorate sensing Potentioselectrode*

The membranes were glued to one end of a pyrex glass tube with Araldite. The tube was then filled with an internal filling solution of 10^{-4} M phorate in 0.1 mM of phosphate-saline buffer. The process of membrane casting and fabrication of biomimetic potentiometric sensor is given in Scheme.3.2. The sensor was kept in air when not in use.

3.4 OPTIMISATION STUDIES

The sensor performance depends a lot on the optimal design of the membrane. The various parameters of phorate sensor membrane have been optimized under similar lines as mentioned in Section 2.4.

3.4.1 Influence of Plasticizer

The response of the sensor strongly depends on the mobility of electroactive species, thereby reducing the resistance. Incorporation of suitable plasticizer influences the working concentration range of potentiometric sensor by enhancing the mobility of target analytes. Moreover, it ensures flexibility to the membrane. Table.3.1 shows the potential output of phorate IPIM sensor with different plasticizers NPOE, BEHS, TEHP and DOP. From the table, it is evident that membranes with NPOE and BEHS alone offers better response characteristics in the concentration range $1x10^{-9}$ to $1x10^{-5}$ M compared to TEHP and DOP based sensors. Also, the magnitude of potential difference for these two membranes is higher in each decade.

Considering the stability of EMF output and consequent better precision of NPOE-based sensor over BEHS, NPOE is preferred for fabricating phorate IPIM sensor. This observation is coherent with earlier [Shamsipur *et al.* 2002; Singh and Saxena, 2005] reports on role of plasticizers which can be attributed to the higher dielectric constant of the plasticizer, i.e. plasticizer NPOE having high dielectric constant of 24.0 giving better characteristics than DOP ($\epsilon = 5.0$), BEHS ($\epsilon = 4.0$) and TEHP ($\epsilon = 4.8$). It is pertinent to mention here that the phorate membranes were found to be brittle in the absence of plasticizer and the sensor performance could not be studied.

Table 3.1 *Potential output of phorate IPIM sensor fabricated using various plasticizers*

[Phorate], M	Potential response (mV/decade)			
	NPOE (ε =24.0)	DOP (ε =5.0)	BEHS (ε =4.0)	TEHP (ε =4.8)
10^{-9} to 10^{-8}	+2	-	+8	-
10^{-8} to 10^{-7}	+5	-	+6	-
10^{-7} to 10^{-6}	+19	+16	+17	+12.0
10^{-6} to 10^{-5}	+41	+32	+35	+28

3.4.2 Effect of PVC to MIP particles ratio

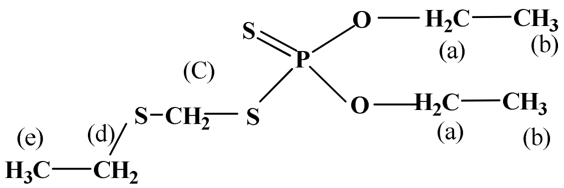
Since the phorate IPIM sensor performance depends heavily on the affinity sites available for template binding, optimum ratio of PVC to MIP is essential. Table 3.2 shows the performance of the membrane sensor while varying the amount of MIP particles in the PVC matrix. From the table, it is clear that the membranes with 1:1 and 2:1 ratios are equally good or rather increasing the amount of MIP particles beyond a particular limit does not enhances its response behaviour in contradiction to our expectation. This may be due to the inhomogeneous nature of the 2:1 membrane thereby restricting the interaction of template with affinity sites. In the case of membranes with 1:0.5 ratio, the total number of binding sites available for the rebinding of phorate is relatively low, for the membrane to respond effectively. Hence, the 1:1 membrane was selected for further studies.

Table.3.2 *Effect of weight ratio of MIP particles to PVC on phorate IPIM sensor response*

Weight of MIP particles (mg)	Weight of PVC (mg)	Weight ratio	Working concentration range (M)	Slope characteristics
45	90	0.5:1	$5x10^{-7} – 10^{-5}$	+ve
90	90	1:1	$5x10^{-9} – 10^{-5}$	+ve
180	90	2:1	$5x10^{-9} – 10^{-5}$	+ve

3.4.3 Effect of pH

The pH of the test solution plays a crucial role in deriving optimum sensor performance. The influence of pH on the phorate IPIM sensor response has been studied by varying it from 8 to 11 in steps of 0.5 after adding 5 mL of 1.0 M Tris-(hydroxymethyl) aminomethane (tris) buffer. The results indicate that the optimum pH for constant and maximum response behaviour over the entire concentration range of phorate ($5x10^{-9}$ to $1x10^{-5}$ M) is 9.5 to 10.5. Hence, the pH of the test solution was adjusted to ~ 10 after the addition of 5 mL of 1.0 M Tris buffer. Phorate exists as neutral molecule even at such alkaline pHs. This is evident from the NMR spectral data of extracted sample which was allowed to equilibrate with the test solution for 5hrs. It clearly matches with the NMR spectra of the pure sample of phorate.



The ^{1}H NMR spectra of the standard phorate in $CDCl_3$ showed a 4H multiplet at δ 4.2 corresponds to 2 CH_2 protons (a), a 6H triplet at δ 1.5 corresponds to 2 identical methyl protons (b), two 2H singlet at δ 4.0 corresponds to CH_2 protons (c), 2 H quartet at δ 2.0 corresponds to CH_2 protons (d), 3H triplet at δ 1.4 corresponds to CH_3 protons (e). The NMR spectra have shown identical peaks as that of the standard phorate sample. Also literature reports are there regarding the degradation of phorate which states that the half life period of phorate at pH 10 is about 33hrs [Hong and Pehkonen, 1998]. This leads to the conclusion that phorate exists as neutral at pH around 10 which is our experimental condition.

3.5. DYNAMIC RESPONSE STUDIES

Dynamic response time is another factor that measures the sensing ability of a sensor. The response time was recorded by varying the phorate concentration in the test solution from $1x10^{-9}$ to $1x10^{-5}$ M (See Fig.3.1). The potential versus time profile for phorate is depicted in Fig.3.1. It shows that 95% of the phorate IPIM sensor response was realized within 5 min and reaches equilibrium response in a time of about 10 min. To evaluate the reversibility of phorate sensor, a similar procedure in opposite direction was studied. The measurements performed in the sequence of high to low concentration (from $1x10^{-5}$ to $1x10^{-9}$ M) indicate that the IPIM sensor was reversible analogous to most of the conventional potentiometric sensors. The sensor has exhibited positive response profile in both directions and it has shown excellent reversibility which makes it suitable for continuous monitoring studies.

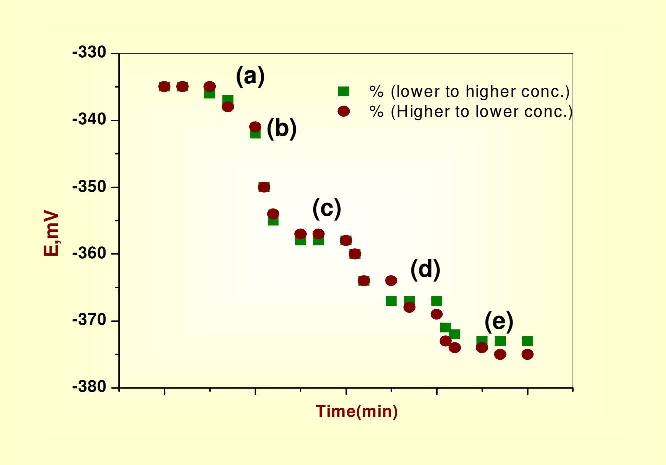


Figure 3.1 *Dynamic response of phorate IPIM based potentiometric sensor a) 1x10^{-9} M; b) 1x10^{-8} M; c) 1x10^{-7} M; d) 1x10^{-6} M; e) 1x10^{-5} M*

3.6 SENSITIVITY AND PRECISION

The response profile of the non-imprinted (NIPIM) and phorate imprinted (IPIM) sensors were compared under the optimum conditions (Fig.3.2).

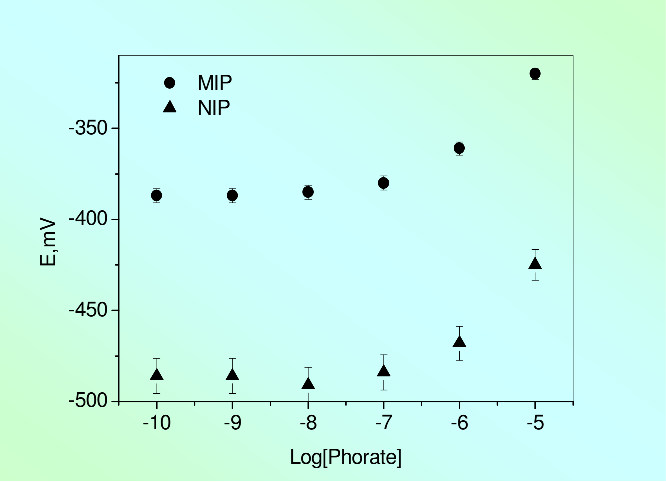


Figure 3.2 *Potential responses of NIPIM and phorate IPIM sensors showing positive slopes*

It was observed that the absolute potentials obtained from non-imprinted polymer materials were unstable (i.e. varying in a range of ± 7mV) which is due to non-specific binding of analyte in contrast to the specific site selective binding in the case of imprinted polymer materials. The stable potential of phorate imprinted membrane is attributed to the imprinting effect. The imprinting effect was further exemplified by the lower detection limit of $5x10^{-9}$ M for imprinted polymer membrane which is calculated based on IUPAC definition [Buck and Lindener, 1994]. On the other hand, the NIPIM do not respond to phorate below $1x10^{-8}$ M and the values were unstable. The sensor has shown positive response in all decades during the molecular recognition of phorate. This anomalous cationic slopes exhibited by the rebinding of phorate into the imprinted cavities can be due to the preferential binding of phorate into the imprinted cavities.

3.7 SELECTIVITY STUDIES

Selectivity is undoubtedly an important issue in the implementation of imprinted polymer inclusion membrane based sensors. In environmental applications, the concentration of the target analytes can be quite low and reasonably high selectivity is essential for effective molecular recognition. Hence, the selectivity of the developed sensor with various common organophosphorous pesticides and herbicides was tested. The potentiometric response results from the exposure to interferences with IPIM and NIPIM-based sensors individually were recorded in the concentration range $1x10^{-7}$ M to $1x10^{-6}$ M and the results obtained are shown in Fig.3.3.

The corresponding response factors (for both MIP & NIP) and subsequent imprinting coefficients presented in Table 3.3 gives a quick appraisal of the superior selectivity of MIP over non imprinted polymers. Response factor corresponds to the ratio of potential difference obtained for the MIP or NIP based sensor for the analyte with respect to that of the interferents and imprinting coefficients corresponds to the ratio of response factors of MIPs with that of NIPs for a particular analyte.

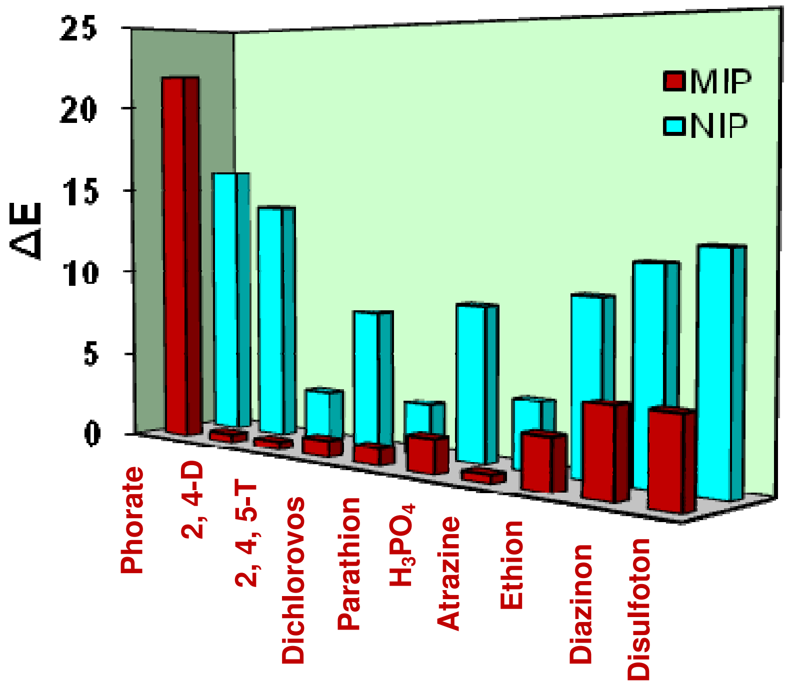


Figure 3.3 *Potentiometric response of phorate IPIM and NIPIM sensor against other pesticides in the concentration range* $1x10^{-7}$ *to* $1x10^{-6}$ *M*

The values depicted in Table 3.3 clearly indicates that the MIP based sensor is far superior selective than the NIP based sensors. In addition this observation proves that the tailor made molecular recognition cavities in MIPs are responsible for the better selectivity compared to that of NIPs. The chemicals which are more likely to coexist with phorate and also interfere gave no false positive readings. The NIPIM sensor has shown lower response to phorate and higher response to interferents than IPIM sensor, due to its non-selectivity which substantiates the imprinting effect. Phorate IPIM sensor responds to diazinon and disulfoton to some extent which can be attributed to the fact that they belong to thioether class.

Table 3.3 *Response factors of the Sensor and its corresponding selectivity coefficients over coexisting analytes*

SL.No	Interferents	Response factor of the sensor for Phorate over various interferents		Imprinting coefficients
		MIP (RF_{MIP})	NIP(RF_{NIP})	= RF_{MIP}/RF_{NIP}
1	2,4-D	44	1.1	38.6
2	2,4,5-T	44	5.3	8.3
3	Dichlorovos	22	2.0	11.0
4	Parathion	22	5.3	4.1
5	H_3PO_4	11	1.8	6.2
6	Atrazine	44	4.0	11.0
7	Ethion	7.3	1.6	4.6
8	Diazinon	4.4	1.3	3.3
9	Disulfoton	4.4	1.2	3.6

3.8 STABILITY AND REUSABILITY

The important criteria required for any sensing device in addition to sensitivity and selectivity is stability and reusability. The above fabricated IPIM based phorate sensor was found to be stable (deviation less than ± 1.0 mV for $5x10^{-9}$ M for phorate) for 3 months and can be reused for more than 40 times without any loss in sensing ability. Furthermore, the sensing membrane does not require any conditioning, i.e. no need for soaking in conditioning solutions after fabrication.

3.9 ANALYTICAL APPLICATIONS

Since the interferents did not give any false positive signals, ground, river and tap water samples were analysed by spiking with known amounts of phorate. The results obtained are shown in Table 3.4 from which it is clear that the IPIM based phorate sensor can reliably be used for monitoring phorate contaminated natural waters containing host of other pesticides and herbicides.

Table.3.4 *Analysis of natural water samples*

SL.No	Sample	Concentration of phorate ($x10^{-9}$ M)		Recovery (%)
		Added	Found	
1	River water (Karamana, TVM)	-	-	-
		2.0	2.05	102.5
		4.0	4.16	104.0
2	Ground water (Trivandrum)	-	-	-
		2.5	2.50	100.0
		5.0	5.10	102.0
3	Tape water	-	-	-
		2.0	2.02	101.0
		5.0	5.10	102.0

*Average of triplicate measurements

3.10. EXPERIMENTAL

3.10.1 Reagents

Phorate, Parathion, Atrazine, Dichlorovos, Ethion, Disulfoton, Diazinon, 2, 4-D, 2, 4, 5-T were obtained from SUPELCO, USA. Methacrylic acid (MAA), Ethylene glycol dimethacrylate (EGDMA), 2, 2'-Azobisisobutyronitrile (AIBN), Di n-octylphthalate (DOP), 2-nitrophenyloctyl ether (NPOE), Bis(2-ethylhexyl) sebacate (BEHS), Tris(2-ethylhexyl) phosphate (TEHP) and high molecular mass poly(vinyl chloride) (PVC) were obtained from Aldrich (Milwaukee,WI, USA). All other reagents used were of analytical grade and deionized water was used throughout.

3.10.2 EMF Measurement

Stock standard solution of phorate (10^{-4} M) was prepared in 10^{-3} M phosphate-saline buffer (10^{-3}M NaH_2PO_4+10^{-3} M KCl). The solutions of 1.0x10^{-5} to 1.0x10^{-9} M of phorate were prepared by aqueous dilution of a definite volume from the stock standard solution by maintaining an overall concentration of 10^{-4} M in NaH_2PO_4 and 10^{-4} M in KCl. The sensor was conditioned in 25 mL of 0.1 M Tris buffer with pH 10 for 30 min. The response of the sensor was examined by measuring the electromotive force (EMF) of the following electrochemical cell.

Ag, AgCl 1.0x10^{-4} M phorate in 0.1 M phosphate saline | PVC membrane | sample || KCl (saturated) | $HgCl_2$, Hg.

The potential response of the sample solutions containing different concentrations [1x10^{-9} M to 1x10^{-5} M] of phorate in 50 mL of 0.1 M Tris buffer (pH 10) was measured. The EMF was plotted as a function of phorate concentration.

3.10.3. Analysis of river/ground/tap waters

The pHs of ground, river or tap water samples (~ 45 mL) were adjusted to 10 after the addition of 5 mL of 1 M tris buffer using HCl or NaOH. The samples were analysed via standard addition method using the above fabricated IPIM-based phorate potentiometric sensor by following the analytical procedure mentioned in section 3.10.2.

3.11. CONCLUSIONS

In this chapter we have described the design and development of imprinted polymer inclusion membrane based potentiometric sensor for phorate, an organophosphorus pesticide. A positive potential change has been observed during the molecular recognition of the neutral analyte at the membrane solution interface. The sensor has exhibited wider working concentration ranges with lower detection limits of $5x10^{-9}$M which is essential for monitoring such environmental toxins. The selectivity of the IPIM is remarkable compared to corresponding non-imprinted polymer inclusion membrane (NIPIM) based potentiometric sensor. Again, the phorate sensor developed in this chapter does not require any conditioning unlike most other potentiometric sensors. The high stability, good reversibility, reusability, portability and absence of memory effect enables the present IPIM based phorate sensor to pave new directions in neutral molecule sensing and thrives us to develop such site selective sensors for other organic pollutants as well.

Chapter 4

Semi-covalent Imprinted Potentioselectrode Employing In situ Polymerized Membranes

4.1 Abstract

Molecularly imprinted polymer membranes was prepared by semi-covalent imprinting strategy wherein i) the template diethyl chlorophosphate (DCP), (a simulant of organophosphorous nerve agents), is covalently linked to the reactive functional monomer vinyl aniline (VA) during imprinting step followed by non-covalent rebinding and ii) in-situ polymerization via single pot synthesis in presence of additional functional monomer, 2-hydroxyethyl methacrylate (HEMA) and crosslinking monomer, ethylene glycol dimethacrylate (EGDMA) after addition of 2-nitrophenyl octyl ether (NPOE) and 2, 2'-azobisisobutyronitrile (AIBN)as plasticizer and initiator respectively. The resulting membrane is integrated with a potentiometric transducer while designing a DCP sensor. The fabricated sensor responds over a wider concentration range of 10^{-6} *–* 10^{-2} *M with a lower detection limit of* 10^{-6}*M. In addition, in-situ monolithic membrane based sensor was designed by adopting non-covalent imprinting strategy also. A detailed comparison is made between semi-covalent and non-covalent in-situ membrane based sensors on the prime sensor performance criteria such as sensitivity, selectivity, working range, response time, reusability and reversibility. Again, the relative merits and demerits of semi-covalent vis-à-vis non-covalent strategy based in-*

situ monolithic membrane sensors were also highlighted. The probable molecular recognition mechanism is also discussed.

4.2 INTRODUCTION

The two main strategies employed in molecular imprinting are covalent and non-covalent [Wulff, 1995]. In covalent strategy, the functional groups are precisely positioned in the active sites and a stoichiometric amount of functional monomers are required. However, the range of template functionality for which efficient reversible complex formation is rather limited. On the other hand, in non-covalent approach, even though the rebinding kinetics is faster, probabilities of non-specific binding is more, which lowers the selectivity considerably. Also in non-covalent method, the in-homogeneity of binding sites is unavoidable due to the formation of multitude of complexes between the functional monomers and template during initial stages of polymerization. Although the notion of combining the advantages of covalent bonds during the imprinting step with those of non-covalent interactions for rebinding was present in earlier work by Wulff [Wulff and Sarhan, 1972; Wulff *et al.* 1973; Lauer *et al.* 1985], the first example of using exclusively covalent interactions for the imprinting step and non-covalent ones for rebinding was reported by Sellergren's group [1990]. Whitcombe *et al.* [1994, 1995] have refined this strategy by proposing sacrificial spacer approach. This strategy termed as "Semi-covalent strategy" is an attempt to synergize the advantages of the covalent (Strict control of functional group location, more uniform distribution) with that of the non-covalent one (reduced kinetic restriction during rebinding). This type of semi-covalent strategy has also been demonstrated by Cheong *et al.* [1997] using carboxylic ester template.

The previous chapters deal with the casting and sensing studies of imprinted polymer inclusion membranes for the selective molecular recognition of methyl phosphonic acid and phorate by non-covalent approach. Various factors influencing the optimum sensing characteristics such as influence of plasticizers, amount of MIP particles, pH etc are described. In both cases the molecular recognition moieties are molecular imprinted polymer particles which are dispersed in a base polymer matrix of PVC.

The development of MIP based membrane technology is at a slow pace as evident from Piletsky's statement "the studies on molecularly imprinted membranes (MIM) are now in their infancy" [Piletsky *et al.* 1999]. Haupt has highlighted this subject in the motto, we quote 'Although MIPs are stable, easy to prepare, and inexpensive, there are still challenges to overcome in their fabrication and use' [Haupt, 2003]. This situation has not changed lately. Sellergren reviewed in the 2002 workshop on Molecularly Imprinted Polymers and Related Technologies hold at La Grande Motte. He counted only 9 of 121 contributions dealing with membrane imprinting [Sellergren, 2003]. These concerns indicate that in imprinted monolith membranes 'there is still a challenge to overcome in the membrane fabrication'. In this chapter we have developed a novel membrane casting format viz. an in situ monolithic membrane for the selective potentiometric sensing of diethyl chlorophosphate via semi-covalent imprinting. One of the major problems related to potentiometric sensors is the absence of a versatile supporting polymer matrix other than PVC and CTA [Nghiem *et al.* 2006]. In the sequential method of IPIM sensors described in the earlier chapters imprinted polymer particles are dispersed in PVC matrix which will have heterogeneity problems, poor distribution of imprinted sites due to insolubility of imprinted polymers.

Hence we have made an attempt to fabricate an in situ potentiometric sensing membrane exclusively of MIP. Hitherto Piletsky and coworkers has demonstrated the application of MIP membrane for sensing application in conductometric sensors [Piletsky *et al.* 1998]. Agostino's group has reported the casting of MIP membrane for potentiometric sensing of atrazine [Agostino *et al.* 2006]. In the present chapter, we describe the synthesis of MIP based in situ membrane for the potentiometric sensing of Diethyl chlorophosphate (DCP), a simulant of Sarin using Semi-covalent and Non-covalent approaches. Inaddition, we have done a comparative evaluation of the sensing characteristics of MIP based sensing membranes based on Semi-covalent and Non-covalent approaches.

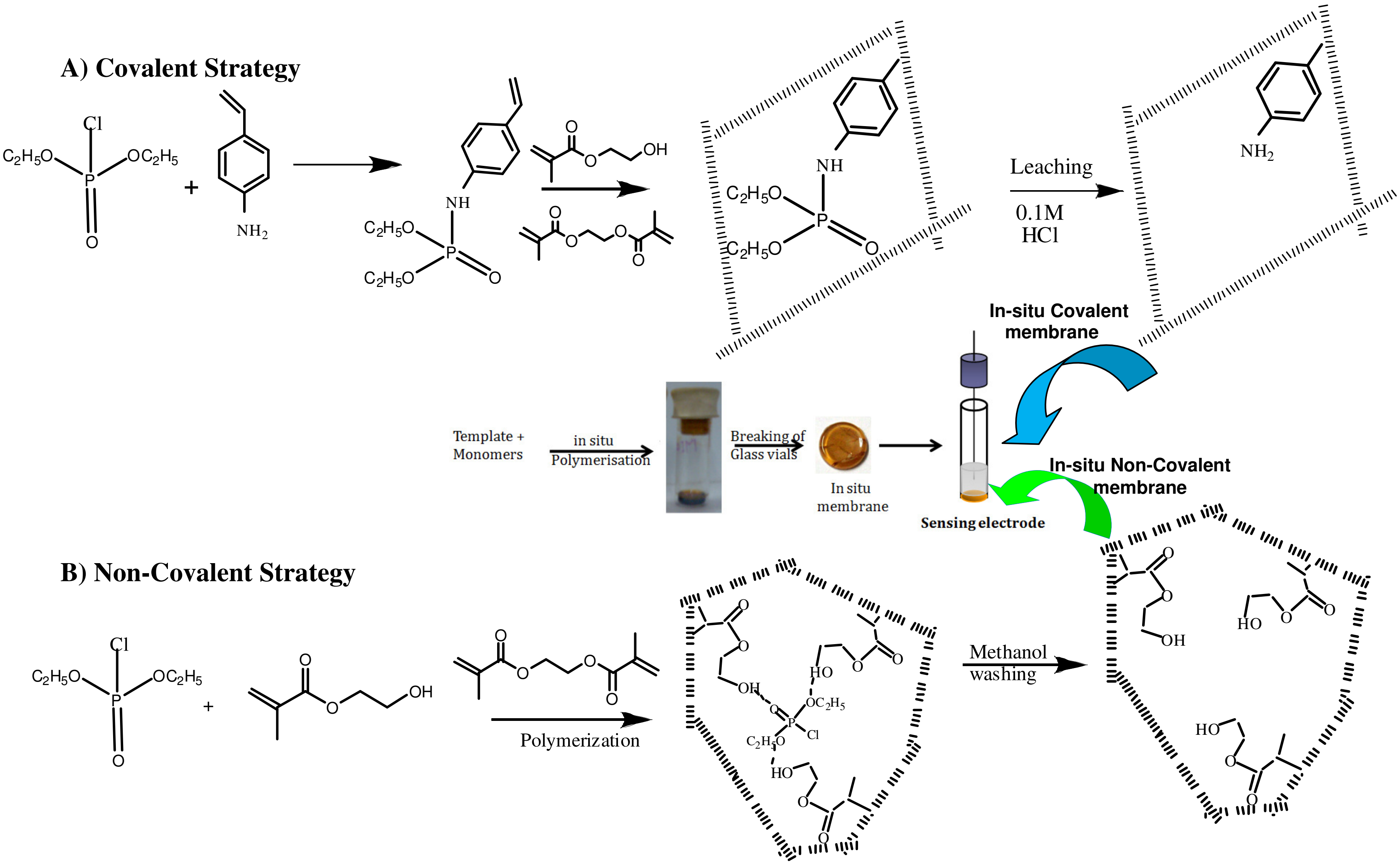
4.3 CONSTRUCTION OF DCP SENSOR

4.3.1. Synthetic Strategies

The two main synthetic strategies viz. covalent and non-covalent were employed to prepare molecularly imprinted polymer recognition membranes by single pot synthesis. The schematic representation of the same is shown in Scheme 4.1

4.3.2. Single Pot Synthesis of Covalent In situ Polymer Membrane

In situ membranes were casted as follows: 0.186 mmol of DCP, 0.186 mmol of 4-Vinyl aniline (VA) to form covalent bond with DCP thus resulting in covalent interactions, 2.26 mmol of HEMA, 0.35 mmol of EGDMA, 0.41 mmol of NPOE and 0.03 g of AIBN were taken in a glass vial of 5 mL capacity, purged with N_2 and sealed under inert atmosphere, placed in a hot air oven maintained at 60°C.



Scheme 4.1 *Synthesis of in-situ biomimetic membrane via (a) covalent and (b) non-covalent strategies and sensor construction*

After polymerization, the vials were taken out from the oven and the polymer membrane formed is taken out by breaking the glass vial. As already described, the corresponding non-imprinted membranes were also synthesized. The plasticizer NPOE is added to the reaction mixture to provide flexibility to the imprinted monolithic membrane, in the absence of which the membranes were found to be brittle and break away during template removal.

4.3.3. Preparation of Non-covalent In situ Membrane

Non-covalent in situ membranes were prepared in a similar manner as that of covalent in situ membrane except the addition of reactive functional monomer vinyl aniline, which is responsible for the covalent linkage with DCP. Hence, the analyte DCP is held non-covalently within the polymer matrix.

4.3.4 Leaching of DCP Template

Preparation of Leached in-situ membranes (covalent or non-covalent)

Covalent in-situ membranes were washed with methanol and leached with 50 mL of 0.1 M HCl for 1 hr. On the otherhand, the non-covalent in-situ membranes were washed free of template by equilibrating for 1hr with 50mL of methanol.

4.3.5 Membrane Rebinding Experiments

The rebinding studies were done in 0.1M Tris buffer (Tris-(hydroxymethyl) aminomethane at pH~10 wherein the DCP hydrolyses to form diethylphosphonate anion [Sega et al. 1997]. Hence, analyte rebinding occurs through non-covalent interactions irrespective of the fact whether imprinting is covalent or non-covalent. The covalent imprinting and subsequent rebinding via non-covalent interactions of in situ monolithic membrane based sensor will further be referred as “Semi-covalent”.

4.4. CHARACTERISATION STUDIES

4.4.1 Visual Detection

The DCP solutions have shown sudden 'turn-on' coloration upon addition of 4-vinyl aniline to the solution (See inset of Fig.4.1). But this detection method was non-selective as it has shown similar color change upon addition of other simulants. One way of improving the selectivity can be by adopting molecular imprinting technology wherein a "molecular memory" is created analogous to antigen-antibody interaction as has been demonstrated previously [Wulff, 1995; Sellergren, 2001;Yan and Ramstrom, 2005]

4.4.2. Spectral elucidation of DCP-VA adduct

The DCP-VA adduct formed in the synthesis recipe of covalent strategy has been characterized by adopting various spectral techniques as described below.

4.4.2.1 UV-Visible Studies

Fig 4.1 shows the UV-visible spectra of methanolic solutions of DCP (A), VA (B), DCP-VA (C) and the polymerization mixture (D). It can be seen from the UV-visible spectra that the characteristic absorption peak of vinyl aniline occurs at 310 nm and on stoichiometric addition of DCP, the absorption peak is shifted to 430 nm. Again, visually one can observe the color changing from colorless to deep yellow (shown in vials). Also, it can be seen from curve D that the polymerizing mixture overlays exactly to that of the DCP-VA confirming the stability of the phosphorylated product even in the pre-polymerization mixture. The stoichiometry of the complex is established as 1:1 by Job's continuous variation plot.

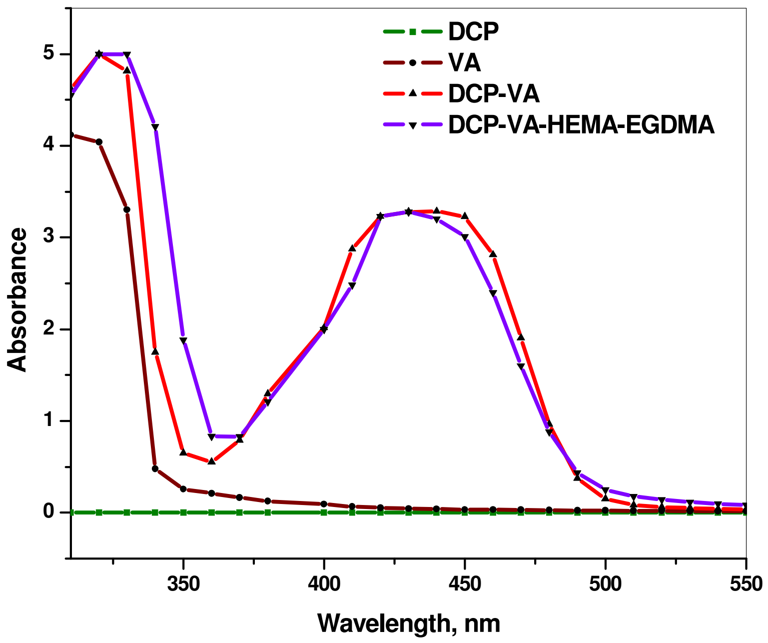


Figure 4.1 *UV-visible spectra of methanolic solutions of diethyl chlorophosphate (DCP), 4-vinyl aniline (VA), DCP + VA and DCP + VA + HEMA + EGDMA against pure methanol respectively (inset:* ***(a)*** *VA alone* ***(b)*** *VA +DCP*

4.4.2.2 FT-IR

IR spectral studies showed that –NH_2 peak present in VA appeared at 3365 cm^{-1} and 3214 cm^{-1}. On addition of DCP, these peaks were shifted to 2861 cm^{-1} and 2576 cm^{-1} respectively, showing the involvement of –NH_2 group in the phosphorylated product formation. Also, we can notice from the IR spectra of DCP and the phosphorylated one, the peaks at 1289 cm^{-1} and 1165 cm^{-1} corresponding to the P=O stretching frequencies were present in both the cases again confirming the formation of DCP - VA adduct [Nakanishi and Solomon, 1977].

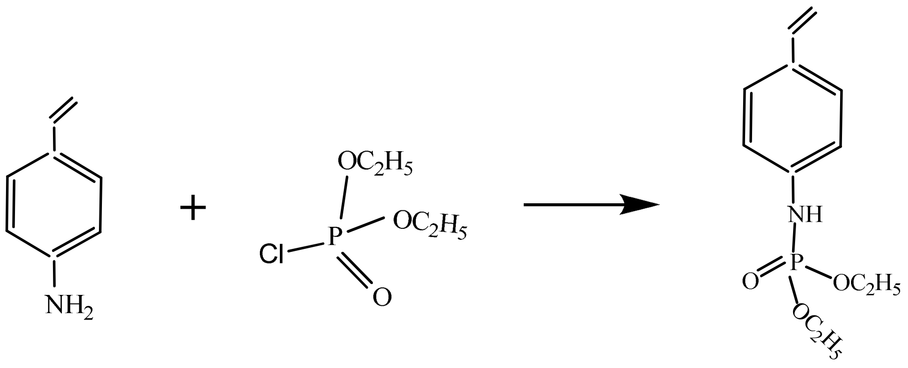
4.4.2.3 NMR

The ^1H NMR spectra of the isolated product in DMSO (d_6) showed a broad band at δ 10.07(1H) corresponding to the – NH proton, a triplet at δ 2.20 (4H) and quartet at δ 4.03 (6H) corresponding to the ethyl protons and a singlet at δ 7.9 (2H) and another singlet at δ 7.5 (2H) corresponding to aromatic protons.

4.4.2.4 Microanalysis of DCP-VA adduct

The micro analysis of the above mentioned DCP-VA phosphorylated product (Shown in Scheme 4.2) after its isolation gave the following results.

Experimental C (62.15%), H (7.71%) and N (6.52%) values are in agreement with theoretical C (64.28%), H (8.0%) and N (6.25%) composition. The agreement between both the experimental and theoretical values is a supportive evidence for the formation of phosphorylated product between DCP and VA.



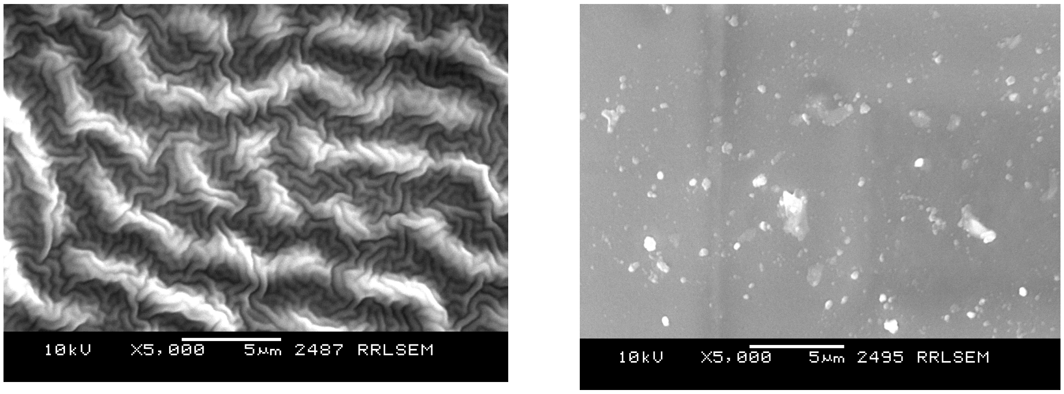
VA DCP DCP-VA

Scheme 4.2 *Structure of DCP-VA phosphorylated product*

Thus, based on UV-Visible, FT-IR, NMR structural elucidation and microanalysis conclusively proves the formation of phosphorylated product by covalent linking of DCP and VA.

4.4.3 Morphological and topographical characterization of recognition membranes

The morphological and topographical characterization studies of semi-covalent and non-covalent in situ monolithic membranes were carried out using scanning electron microscopy. In semi-covalent membrane, particles are uniformly stacked and arranged in a lamellar fashion (See Fig.4.2a). This will result in better host-guest interactions that are essential for effective molecular recognition. The average diameter of the wire like structure is about 200-300nm. The significant increase in sensitivity and selectivity should be attributed to the higher number of surface imprinted sites and large surface to volume ratios.



a **b**

Figure 4.2 *SEM photographs of semi-covalent in-situ (a) and non-covalent in-situ (b) membranes.*

A greater number of effective imprinted sites are available at the surface or in the proximity of the surface of these one dimensional imprinted wires [Xie *et al.* 2006]. On the other hand, the non-covalent in-situ membrane (See Fig.4.2b) shows no such regular arrangement; rather it appears as a closely packed amorphous membrane. The reduced availability of the imprinted sites on the surface of non-covalent membrane culminates for its higher limit of detection and poor selectivity compared to the former one.

4.5 OPTIMISATION STUDIES

4.5.1 Effect of Plasticizer

One of the challenging tasks ahead of us in this study was the in situ synthesis of imprinted polymer membrane. Membrane should be flexible enough so that it can withstand the mechanical stress applied during the breakage of the glass vials, since small leakage in the membrane during sensing studies can plague the response. For this purpose we have varied the template: monomer: cross-linking monomer ratio from the usual 1:8:32 to 1:12:2 ratios. As higher amount of cross-linker makes the polymer highly rigid, thereby making it brittle, we have reduced the ratio of cross-linker in the polymer recipe. But even after reducing the cross-linker, polymers were found to be brittle & we were facing the problem of low turnover rate of the synthesized membranes. Hence we have added plasticizer NPOE, which was found to be the best solvent mediator in our earlier studies, in order to increase the flexibility of the membranes. Addition of 0.41mmols of NPOE during the in situ membrane preparation has improved the flexibility of the membranes and we were able to eject out the membranes much easier.

4.5.2 Effect of pH

The pH of the test solution plays a crucial role in deriving optimum sensor performance. Effect of pH per decade concentration change ie. 10^{-5} to 10^{-4} M has been studied by varying it from 4 to 12 after addition of 5mL of 1.0 M tris (hydroxymethyl) aminomethane (Tris) buffer. The results indicate that the optimum pH for constant and maximum response was found to be 10.0 to 11.0. This is due to the formation of diethylphosphonate anion at pH’s<11.0 and >10.0. The response decreases for both semi-covalent and non-covalent membranes on either side of the optimal pH range. Hence, the pH of the test solution was adjusted to 10.5 ± 0.5 after the addition of 5 mL of 1.0 M Tris buffer.

4.5.3 Effect of Temperature

The effect of temperature on the response characteristics of the sensor over a temperature range of 30-50 ^{0}C was studied. The response profile of the sensor at 30 ^{0}C, 40 ^{0}C and 50 ^{0}C has been shown in Fig.4.3a, b and c respectively.

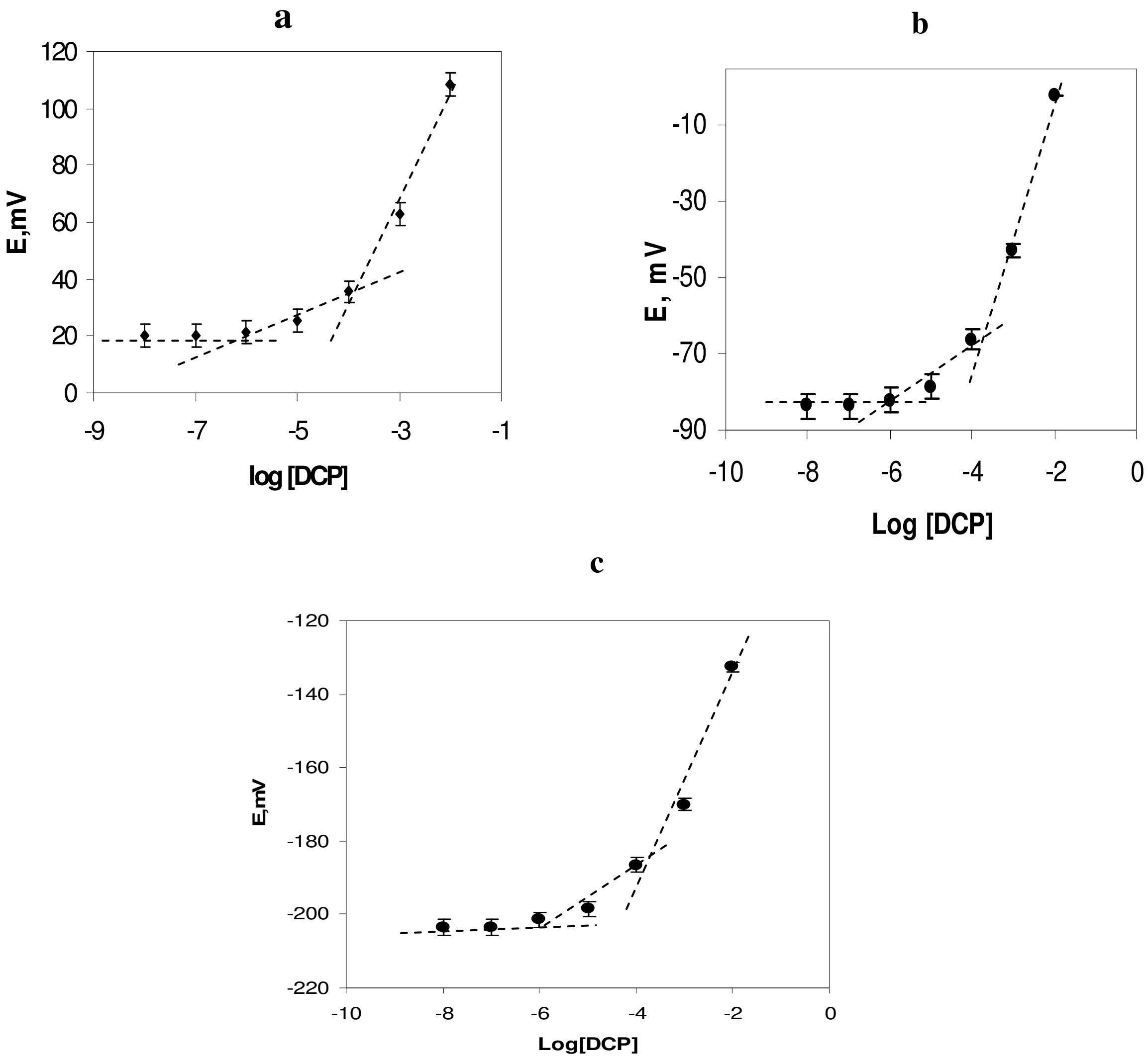


Figure 4.3 *Potential response of biomimetic potentiometric sensor employing in situ semi- covalent strategy for DCP MIP at (a) 30^{0}C at (b) 40^{0}C and at (c) 50^{0}C respectively.*

It was found that temperature influences the response characteristics of the in situ imprinted sensor membrane. Even though the sensitivity is not affected, the magnitude of potential change per decade was found to decrease as the temperature increases. The decrease may be due to deviation from usual synthesis protocol where cross-linking monomers are used in higher proportions which are responsible for keeping the recognition cavity intact under harsh conditions. But it was found that as the cross-linker was increased, the membrane became brittle which was undesirable for sensing purpose. More detailed studies are required to investigate the influence of temperature and the plausible ways to increase the cross-linking density without affecting the membrane characteristics.

4.6 RESPONSE TIME

The sensor exhibits the equilibrium response within 2 minutes viz. semi-covalent and non-covalent, which is much faster in comparison with other conventional potentiometric sensors for organic molecules, where it takes even 10 minutes in some cases to attain the equilibrium response [Kitade *et al.* 2004]. On the other hand, the NIPs require more than 5 minutes for attaining an equilibrium response, which is due to the absence of a specific complementary cavity.

4.7 SENSITIVITY & PRECISION

The sensing performance of semi-covalent and non-covalent in situ membranes [MIP and NIP] has been systematically investigated. Fig.4a & 4b shows the response characteristics of semi-covalent in-situ membranes, viz. MIP and NIP and Fig 4c & 4d that of non-covalent ones respectively.

From the figure, it is clear that the semi-covalent sensor responds over a wide dynamic concentration range of $10^{-6} – 10^{-2}$ M of DCP with a detection limit of $1x10^{-6}$ M. Under optimum conditions described above, the calibration curve was linear in the concentration range$1x10^{-6}$-$1x10^{-4}$ and$1x10^{-4}$-$1x10^{-2}$ M of DCP.

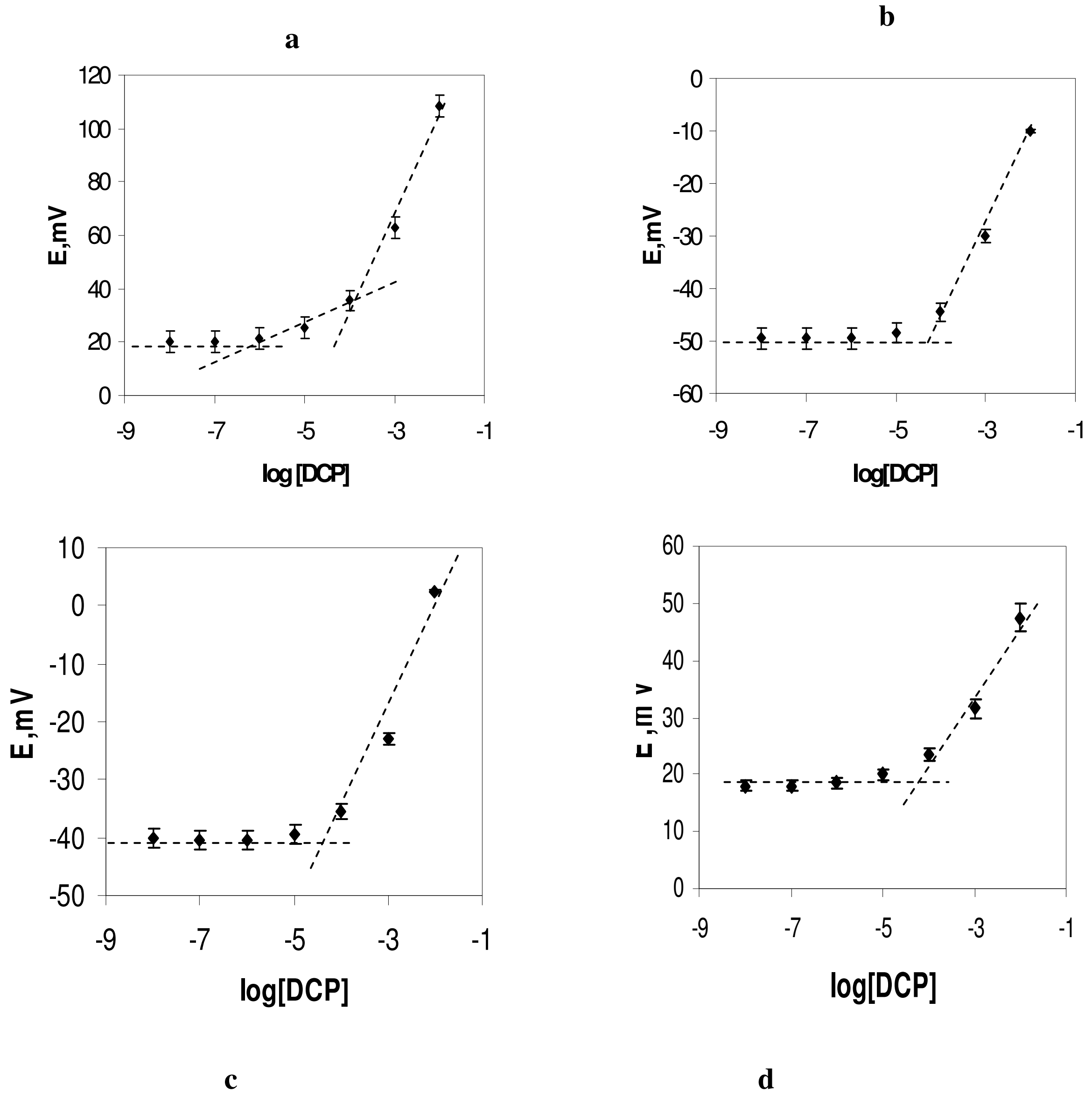


Figure 4.4 *Potential response of biomimetic potentiometric sensors employing in situ membrane formats prepared by semi-covalent strategy for DCP (a) MIP (b) NIP and non-covalent strategy (c) MIP (d) NIP at 30 ±1^{0}C respectively.*

The linear regression equations are as follows:

E = +36.5C + 178.5 & E = +7C + 62.5 ------------- (1) (Semi-covalent MIP)

E = +17.25C+ 23.58 ----------------------------------- (2) (Semi-covalent NIP)

The corresponding linear ranges and limit of detection for NIPs are 10^{-5} – 10^{-2} M and $1x10^{-4}$ M respectively (See Fig.4b). The lower detection limits and wider working range in case of MIP over NIP is a clear indication of the imprinting effect. The NIPs have shown slight response due to the non-specific binding of analytes.

Fig 4c & 4d shows the response characteristics of non-covalent MIP & NIP respectively. From the figure it is clear that the non-covalent membrane responds in the range 10^{-5} to 10^{-2}M with a detection limit of 1x 10^{-4}M. The linear regression equations are as follows

$E = + 19C + 38.33$ --------------- (3) (Non-covalent MIP)

$E = + 12C+70.17$ ------------- (4) (Non-covalent NIP)

We have observed positive slopes in all types of DCP sensor described in this chapter in a similar vein of the response characteristics reported in the last two chapters.

4.8 SELECTIVITY

The sensor should preferably have high degree of selectivity so that it can distinguish the target analyte when coexist with a host of other interferents. The selectivities of the various in situ membranes casted were carried out to investigate this aspect. The interferents investigated include PMP (simulant of Soman), MPA (nerve agent's ultimate degradation product), pesticides like phorate and 2, 4-D and acetic anhydride.

Fig 4.5a & b shows the change in potential on increasing the DCP concentration from 10^{-5} – 10^{-4} M for semi-covalent & non-covalent in situ membranes (both MIP and NIP) respectively. From the figure, it is clear that the semi-covalent MIP selectively recognizes DCP far ahead in comparison with other interferents, whereas the corresponding NIP responded non-specifically.

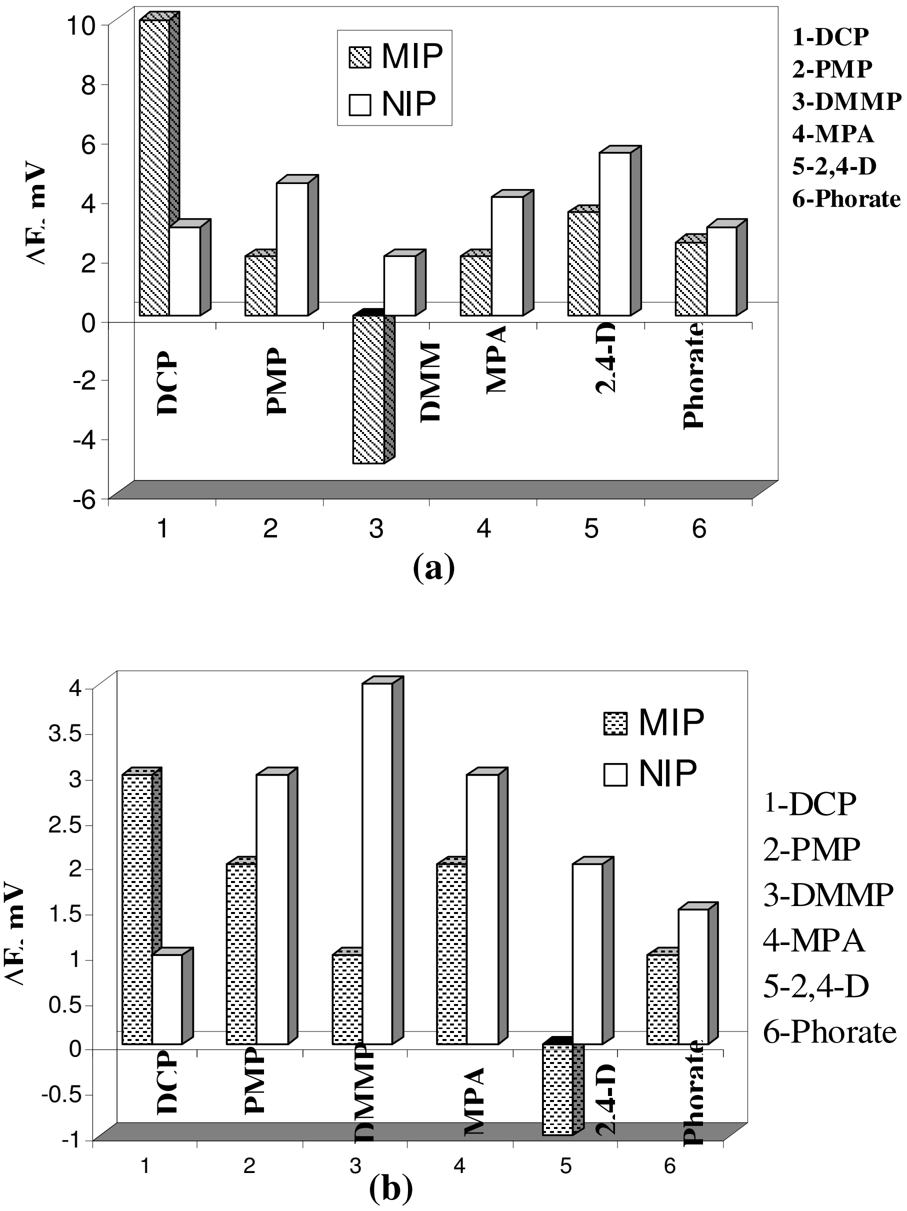


Figure 4.5 *Potentiometric response signal of the MIP or NIP based DCP sensors to DCP and simulants/degradation products of CWA and pesticides for a decade change of concentration from 1x10*$^{-5}$ *M to 1x10*$^{-4}$ *M. (a) in situ semi-covalent (b) in situ non-covalent*

The selectivity coefficients determined by adopting the procedure described elsewhere [Umezawa *et al.* 1995] are shown in Table 4.1 to have a quick appraisal of imprinting effect. The selectivity coefficients are directly related to the complexing properties of lipophilic ligand incorporated into the polymer membrane phase. The covalently immobilized ligand vinyl aniline, selectively rebinds with DCP due to the 'foot print' cavity impregnated in the MIP whereas in NIP such a selective site is absent. The potential change of the NIP may be caused by non-specific binding to the amino group on the surface of the non-imprinted polymer, adsorption and distribution to the sensing element or both [Kitade *et al.* 2004].

From Fig 4.5b, it is seen that eventhough non-covalent MIP response is high for the analyte, the coexisting analytes also gives substantial response reducing the imprinting effect. This is due to more non-specific binding of analytes in the non-covalent mode. Table.4.2 shows a comparison of the selectivity coefficients of DCP over selected interferents using semi-covalent and non-covalent imprinted polymer membranes, which indicates a better selectivity for the former one compared to the latter one.

Table 4.1 *Selectivity coefficients obtained with semicovalent in situ MIP and NIP membrane formats for DCP over selected interferents*

Interferents	Potentiometric Selectivity Coefficient, Kpot $_{A,B}$	
	Semicovalent MIP (x 10^{-3})	Semicovalent NIP (x 10^{-2})
PMP	2.5	1.0
MPA	5.0	1.0
2,4-D	2.5	1.0
DMMP	0.5	1.0
Acetic anhydride	2.5	5.0
Phorate	10.0	2.0

A= DCP, B = interferent

Table 4.2 *Comparison of selectivity coefficient of DCP over selected interferents using Semi-covalent and non-covalent in situ membranes*

Interferents	Potentiometric Selectivity Coefficient, Kpot $_{A,B}$	
	Semicovalent MIP ($x10^{-3}$)	Non-covalent MIP ($x\ 10^{-2}$)
PMP	2.5	20.0
MPA	5.0	3.3
2,4-D	2.5	1.6
DMMP	0.5	3.3
Acetic anhydride	2.5	1.1
Phorate	10.0	5.0

A= DCP, B = interferent

4.9 REUSABILITY & REVERSIBILITY

The semi-covalent and non-covalent sensors when stored in air are stable up to 3 months and can be reused for more than 20 trials. Again, the sensor outputs were reversible in the whole concentration range, i.e. from higher to lower and vice-versa.

4.10 COMPARISON OF SEMI-COVALENT VIS-A-VIS NON-COVALENT STRATEGY

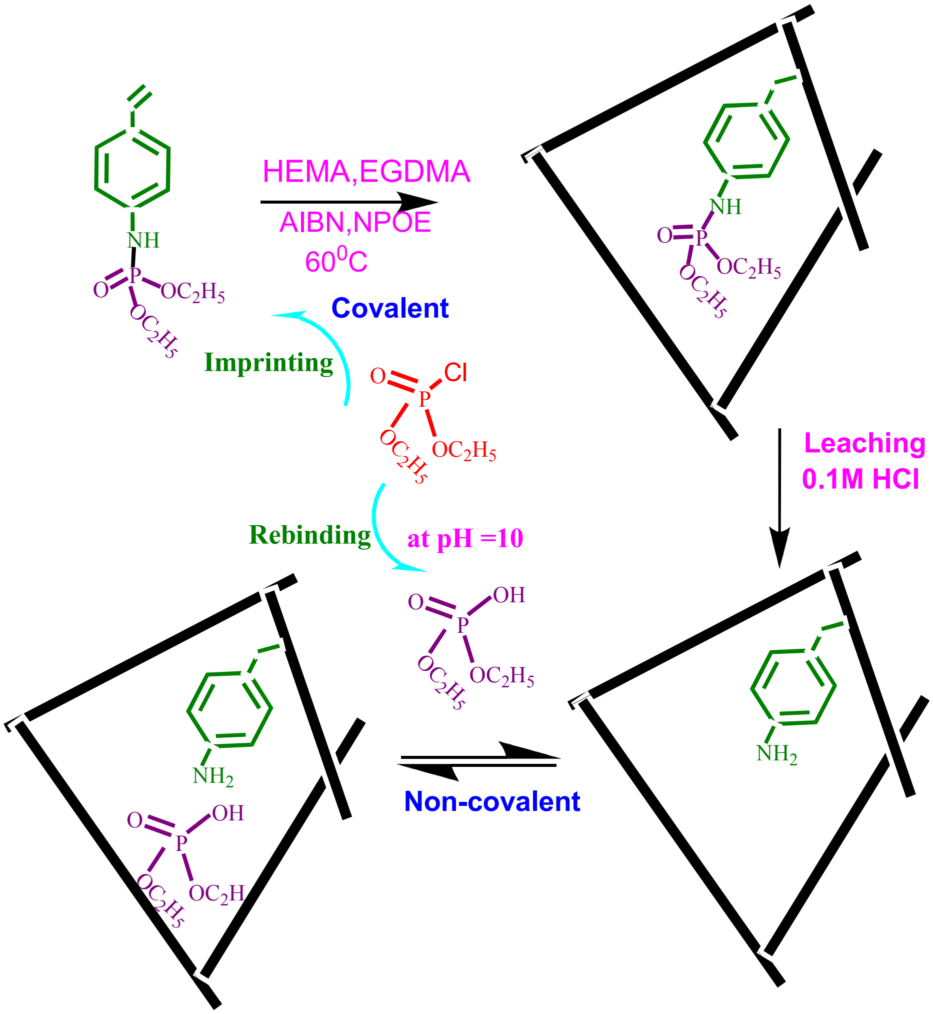
The response curves for semi-covalent and non-covalent in situ imprinted polymer membranes (MIP) are shown in Figs.4.4 (a) and 4.4 (c) respectively. From the figure it can be seen that the dynamic working concentration range is $10^{-6} – 10^{-2}$ M for semi-covalent mode (Fig.4.4a), whereas it is $10^{-5} – 10^{-2}$ M for non-covalent mode (Fig.4.4c). Also the magnitude of response, i.e. ΔE value is higher for semi-covalent compared to non-covalent strategy. The detection limit is lower ($1x10^{-6}$ M) in the case of semi-covalent (0.17ppm) as compared to that of non-covalent (1.7ppm). The selectivity of DCP sensor based on semi-covalent strategy is superior to non-covalent as the potentiometric selectivity coefficients ($K^{pot}_{A,B}$) are lower in case of former (see Table 4.2). However, both the sensors are stable and exhibits analogous response times of 2 min. Moreover sensors prepared using both strategies have displayed reversibility and reusability, essential for continuous monitoring studies.

4.11 RESPONSE MECHANISM

The development of new host-guest interaction with a function of molecular recognition involving signal transduction is a challenging problem from the fundamental aspect of designing and synthesizing molecules with tailored functions as well as their application to new type of materials including those of analytical use. In the present work, the analyte DCP is covalently anchored to the reactive functional monomer vinyl aniline in case of semi-covalent DCP sensor. During leaching (hydrolysis) using HCl, the analyte is stripped off as diethyl phosphonic acid. So the cavity formed in the polymer matrix on leaching covalent in situ monolithic membrane presumably corresponds to that of diethyl phosphonic acid (See Scheme 4.3). During the rebinding process, which is done at pH 10.5, the analyte DCP when added to the sample solution containing Tris-buffer will undergo hydrolysis to form diethylphosphonic acid [Sega *et al.* 1997], which is same as that of the leached template. Hence, it can be construed that template rebinding takes place by non-covalent interactions. The proposed rebinding mechanism is as shown in Scheme 4.3.

In the case of non-covalently imprinted in-situ membrane, when template removal is carried out using methanol, DCP molecules incorporated inside the imprinted sites will be removed and the cavities corresponds to that of DCP are created.

But in Tris buffered (pH 10.5) sample solution, the analyte is in the hydrolysed form. So, effective rebinding will not take place in non-covalent strategy based DCP sensor. This explains the better sensitivity, lower detection limits and higher magnitude of potential change in each decade for semi-covalent strategy based potentiometric sensor over non-covalent one. Furthermore, in semi-covalent approach, the covalent linkage of DCP template with reactive functional monomer – vinyl aniline prior to imprinted polymer material synthesis reduces the contributions of non-specific interactions to a large extent unlike non-covalent one resulting in better selectivity over the latter.



Scheme 4.3 *Rebinding mechanism by semi-covalent approach*

4.12 ANALYTICAL APPLICATIONS

Since the interferents did not give any false positive signals for semi-covalent strategy based in-situ sensor, ground water samples were analysed by spiking known amounts of DCP and varying concentrations of interferent mixtures. The results obtained are shown in Table 4.3. It is clear from the table that the recovery on addition of known amounts of DCP to ground water was found to be quantitative demonstrating the feasibility of the developed sensor for routine monitoring of DCP in natural waters containing host of other organophosphorous chemical warfare agent simulants and pesticides.

Table 4.3 *Analysis of ground water samples*[a]

SL. No	[DCP] , M		Recovery (%)
	Added (x 10^{-5} M)	Found [c] (x 10^{-5} M)	
1	1.0	0.98 ± 0.07	98.0
2	0.5[b]	0.48 ± 0.07	96.0
3	1.0 [b]	1.01 ± 0.03	101.0

[a] In presence of 1×10^{-5}M each of PMP, MPA, Phorate and 2,4-D.
[b] Volume of sample = 50mL

4.13. EXPERIMENTAL

4.13.1 Reagents

Diethyl chlorophosphate (DCP), 4-Vinyl aniline (VA), 2-Hydroxyethyl methacrylate (HEMA), Methyl methacrylate, Dimethyl methyl phosphonate (DMMP), Pinacolyl methyl phosphonate (PMP), Ethylene glycol dimethacrylate and 2,2'-Azobisisobutyronitrile, 2-nitrophenyloctyl ether (NPOE), Methyl phosphonic acid, Acetic anhydride and 2, 4-D were purchased from Aldrich (Milwauke, WI, USA). Phorate was obtained from SUPELCO, Pennsylvania, USA. All other chemicals including NaH_2PO_4, H_3PO_4 and Tris-buffer were of analytical reagent grade obtained from E-Merck, India.

4.13.2 Equipments

Potentiometric response characteristics were studied with an ELICO make Ion analyzer, Model No. LI 126 (ELICO,Hyderabad, India). UV-Visible absorbance spectra were recorded with a Shimadzu-UV-2401 PC controlled double beam Spectrophotometer (Shimadzu, Japan). NMR spectra were recorded with DPX-300 NMR Spectrometer (Bruker Avance, Switzerland). The elemental analysis was carried out using Perkin Elmer Elemental Analyzer, Model 2400 (Perkin Elmer, Switzerland), FTIR spectra were recorded in the range 4000-400 cm^{-1} using Spectrum One FTIR Spectrometer (Perkin Elmer, Switzerland). The surface morphology of the imprinted membranes was studied using scanning electron microscope (SEM) (JEOL, Model JSM 5600 LV).

4.13.3 EMF Measurements

The semi-covalent and non-covalent in situ membranes were glued to one end of a pyrex glass tube. The tube was then filled with 1mL of internal filling solution of 10^{-3} M DCP. The measurements were carried out with respect to saturated calomel electrode (SCE) as the reference electrode with the following cell assembly Ag/AgCl/10^{-3}M DCP, in situ membrane‖sample solution ‖ Hg, $HgCl_2$, KCl (Saturated). The sensors were kept in air when not in use.

All measurements were made in 50 mL of 0.1 M Tris buffer (pH=10) and the electrochemical cell is equipped with a magnetic stirrer. The response of the sensors was examined by measuring the electromotive force (EMF) of the electrochemical cell described above. The potential responses of the solution containing 10^{-8} to 10^{-2} M amounts of DCP in 50 mL of 0.1 M Tris buffer was measured and obtained EMF values were plotted as a function of DCP concentration.

4. 14. CONCLUSIONS

In the present study we have demonstrated how the imprinting and rebinding strategies can be tuned so as to get the optimum sensing performance. This was the first report on the utilization of semi-covalent imprinting strategy for potentiometric sensing applications. During the study we have compared the performance of the semi-covalently imprinted sensor with that of non-covalently imprinted sensor, a versatile strategy in MIPs and found that the semi-covalent one has amalgamated the advantages of covalent and non-covalent imprinting viz. better site selectivity and easy rebinding. Moreover, the utility of in situ monolithic imprinted polymer membrane based on semi-covalent approach for design and development of potentiometric transducer based sensor is clearly brought out. The synchronization of molecular imprinting and membrane formation during sensor construction has been done in a single step, thereby avoiding heterogeneous nature of conventional ISE's and imprinted polymer inclusion membrane sensors. Again, the methodology possibly precludes the post modification of imprinted polymer to isolate "Good sites" from "bad sites" –the major bottleneck in MIPs as the membrane is uniform as seen from SEM micrograph of the semi-covalent in situ monolithic imprinted polymer membrane.

Chapter 5

Ion Associates as Optical Molecular Probes

5.1 INTRODUCTION

Metals and their ions are ubiquitous in nature. Heavy metals are an important class of pollutants with both lethal and sub lethal effects on organisms. Human activities have modified and interfered with natural cycles and caused a release to the aquatic and terrestrial systems. Besides the fact that some heavy metal (HMs) ions are essential to many organisms in small doses, high doses affect ecosystem and human health, and in the case of very toxic metals even small doses. An extensive survey on metals in the environment has been given by Merian *et al.* [2004]. Heavy metals are defined as metals of density higher than 5g/cm^3. They occur as pure elements, as ions and complexes. Heavy metals were brought into the environment by human activities, which started more than 4000 years ago with metal mining. Unprecedented pollution came up with the industrialization and its consumption of energy. They are second to pesticides, in terms of environmental toxicity. The combustion of fossil fuels introduces a large amount of heavy metals into the atmosphere and the aquatic environment. Crude oil, for example, contains 3.4 ppm mercury and the firing of coal causes the worldwide emission of 2.4 x 10^4 tons of lead per year [Merian *et al.* 2004]. Additionally, heavy metals are released to the ecosystem with the exponential growth of metal mining, the following processes and their industrial use. A further area of concern is in dealing with waste from computer and IT equipment, one of the categories of waste undergoing the most rapid growth worldwide. Such refuse can release copper, lead, mercury and cadmium into the environment. Improper disposal of computer equipment is causing severe problems. In the worst cases, waste has ended up in poorly managed facilities, such as the group of waste sites in Guiyu, south-eastern China, which became the focus of international attention in 2002. At Guiyu, imported computer waste was being picked apart by hand, alongside rivers and fields, creating significant health and environmental risks [Guo *et al.* 2009]. Recently, such incidents of dumping of electronic wastes were reported from Cochin, the industrial capital of Kerala.

Trace metals such as lead, cadmium and mercury do not play an essential role in daily maintenance, but adversely affect an organism if available at toxic levels. Metal toxicity generally occurs via mechanisms such as blockage of essential functional groups of biomolecules (proteins and enzymes), displacement of the essential metal in biomolecules and modification of the active conformation of biomolecules. Heavy metals show a large tendency to form complexes, especially with nitrogen, sulphur and oxygen containing ligands of biological matter [Sigg and Stumm, 1996]. Of the heavy metals released from various products and processes, cadmium, lead and mercury are of greatest concern to human health, because of their toxicity, potential to cause damage at low concentrations, and ability to bio-accumulate [Oehme and Wolfbeis, 1997]. Table 5.1 shows the maximum permissible limits of these heavy metal toxins set by USEPA, their potential health risks and major sources of contamination. Cadmium, exposure occurs mainly through food crops grown on contaminated soils, accumulates in the kidneys and is implicated in a range of kidney diseases [Smith *et al.* 1997]. It may also have important carcinogenic effects and there is reasonable chance for genotoxicity [Boyd,2010].

Table 5.1 Standards and guidelines of heavy metals in drinking water [Wigginton et al. 2007]

Metal	USEPA (mgL^{-1})	Potential Health Effects from Long-Term Exposure Above the MCL	Sources of Contaminant in Drinking Water
As	0.010	Skin damage or problems with circulatory systems, and may have increased risk of getting cancer	Erosion of natural deposits; runoff from orchards, runoff from glass & electronics production wastes
Cd	0.005	Kidney damage	Corrosion of galvanized pipes; erosion of natural deposits; discharge from metal refineries; runoff from waste batteries and paints
Pb	0.015	Delays physical or mental development of children, Cause kidney problems; high blood pressure etc in adults	Corrosion of household plumbing systems; erosion of natural deposits
Hg	0.002	Kidney damage	Erosion of natural deposits; discharge from refineries and factories; runoff from landfills and croplands
Cu	1.3	Gastrointestinal distress, Liver or kidney damages, Cause Wilson's Disease	Corrosion of household plumbing systems; erosion of natural deposits

Mercury, which is found mainly as elemental and methyl mercury, is particularly harmful to foetal and embryonic nervous systems, causing learning difficulties, poor memory and shortened attention spans [Jorgensen *et al.* 1997]. Methyl mercury is also classified as a possible human carcinogen by the International Agency on the Risks of Cancer. Lead is highly persistent and bioaccumulates in the environment. Children under the age six are especially at risk to lead poisoning and may have retarded growth and development. It has strong adhesion property towards soil and remains at upper soil layer like other heavy metal toxins mentioned above. Hence these heavy metals can be classified as “Persistent Inorganic Pollutants (PIPs)” [Gailer, 2007] as they are even more persistent in various forms in nature compared to POPs. Several analytical methods are in use to control and determine HM levels in various matrices. Of those, sensors have attracted substantial interest and are especially advantageous for field, in situ, and remote applications.

5.2 CONVENTIONAL METHODS FOR THE DETERMINATION OF HEAVY METALS

In the past, traditional methods have been used to remediate, control and minimize heavy metal pollutants. The determination of heavy metals is a challenging subject for analytical chemists regarding concentration ranges set by standards and guidelines for reasons of toxicity. In addition, similar chemistry of these metals is fastidious with respect to selectivity of determination method. A number of popular current methods for detecting the presence of heavy metals require somewhat sophisticated analytical techniques. Recommended procedures for the detection of heavy metals in water samples include photometric methods, flame or graphite furnace atomic absorption spectroscopy (AAS), inductively coupled plasma emission or mass spectrometry (ICP-ES, ICP-MS), mass spectroscopy (MS), X-ray fluorescence spectroscopy (XRF) total reflection X-Ray fluorimetry (TXRF) and anodic stripping voltammetry (ASV) [Merian *et al.* 2004; Förstner and Wittmann, 1981; Fresenius *et al.* 1988; Klockenkämpfer, 1997; Sansoni, 1987]. While AAS and photometry are single element methods, ICP-ES, ICP-MS and TXRF are used for multi-element analysis, and voltammetry is an oligo-element approach.

These methods mostly physical or chemical techniques offer good limits of detection and wide linear ranges, but require high cost analytical instruments developed for the use in the laboratories. Although these methods are very precise, even in ppb and ppt range, they are cumbersome and impractical for on-site testing and often expensive to run. The necessary collection transportation and pretreatment of a sample is time consuming and a potential source of error [Spichiger-Keller, 1998]. On the other hand, the last few years have seen a growing development of chemical sensors for a variety of applications. The toxicity of heavy metals makes a continuous supervision of drinking or ground water and lentic or lotic water courses necessary. Chemical sensors are useful alternative tool enabling on-line and field monitoring studies [Wolfbeis, 1991].

Optical chemical sensors are capable of continuous recording of chemical species and thus have found (and will find) numerous applications in areas such as the chemical industry, biotechnology and medicine [Wolfbeis, 2005]. Optical chemical sensors and biosensors may be divided into several subgroups depending on the working principles applied. In fact, practically all spectroscopic methods including absorptiometry, reflectometry, fluorescence (with its numerous parameters including decay time, energy transfer and quenching efficiency in addition to the more conventional measurement of intensity or polarization), infrared and Raman spectroscopies, but also by interferometry and surface Plasmon resonance have been applied, often in combination with fibre optic waveguides. Absorptiometric or colorimetric methods are still preferable and invaluable for their simplicity, sensitivity, selectivity and low operational costs in addition to the possibility of field use.

Optical ion sensing, however, has its limitations. Despite the availability of selective reagents, many optical indicators react with more than one metal ion, whereby selectivity can often be enhanced by proper adjustment of pH. Due to the fact that many metal ion indicators also combine with hydrogen ion, pH has to be controlled or measured so that an appropriate correction factor can be applied [Seitz and Wolfbeis, 1991]. Active molecules that interact with important analytes such as transition metal ions in presence of huge amount of major metals, such as alkaline or alkaline earth ions, through the enhancement of any particular property, are very appealing for analytical and environmental applications.

5.3 ION ASSOCIATES AS OPTICAL MOLECULAR PROBES

Study of the properties of ions in solutions has been a central preoccupation of researchers from the time of the discoveries made by the German physicist-chemist Wilhelm Ostwald and the Swiss scientist Alfred Werner. At the end of the 19th century, Ostwald formulated the basic principles of the theory of color indicators and proposed to consider analytical reactions in water as interaction between ions. Since the majority of chemical reactions (including those in nature) proceed in aqueous solutions, the end of the 19th and the beginning of the 20th centuries can be called the epoch of the origin of one of the most interesting trends of inorganic chemistry - investigation of the properties of colored substances and of their interactions in liquid media, first of all, in water; this trend is based on a surprisingly fruitful symbiosis of the coordination chemistry of inorganic compounds and the physicochemistry of the solutions of organic dyes[Ishchenko and Shapovalov, 2004]

The Werner theory considers the interaction of complex particles from the viewpoint of the processes of complexing. In water (or another solvent), ions exist in the form of aquacomplexes (solvocomplexes), but different ions exist as hydroxoaqua-, halogenaqua-, and oxoaqua complexes and the like — in other matrices. Complexes are considered as the mean combination of particles where there is a direct chemical coupling between the constituent parts, the free energy of the formation of which is greater than the kinetic energy of the solvent molecules at a given temperature. However, a chemical bond does not always arise in an ionic compound. Such kinds of compounds in which there is no direct bonding between the ions are called outer-sphere complexes of the type of ion pairs or, more briefly, ion associates [Kuznetsov, 1986; Lewis and Wilkins, 1963].

According to Werner’s theory, complex ions are coordinationally saturated which are capable of further attracting not only oppositely charged ions but also polar molecules. Contrary to the assumption on the negligibly small influence of outer-sphere ions on complexing in solutions, it was shown that the strength of outer-sphere bonds in a number of complex compounds is commensurable with the strength of the bonds of inner-sphere ones and can even be higher. This was noted not only for concentrated aqueous solutions of salts but also for diluted media. It became clear that there were no grounds for ignoring the "outer-sphere" interactions [Ishchenko and Shapovalov, 2004; Bjerrum,1950; 1957; Monk,1952].

Investigations of the ion associates in the composition of which there is a colored organic ion have undergone particular development. These associates were most widely studied in chemical analysis for developing effective techniques to quantitatively estimate the content of metals, inorganic anions, and many classes of organic substances, including surfactants (SA) in ionic solutions. It was established that the majority of the reactions of formation of complex compounds followed the type of displacement (substitution): $\mathrm{MY}n + m\mathrm{Z} \rightleftarrows \mathrm{MZ}m + n\mathrm{Y}$
or addition: $\mathrm{MY}n + m\mathrm{Z} \rightleftarrows \mathrm{MY}n\mathrm{Z}m$, where M is the central ion and Y and Z are the coordinated ions or molecules (without indication of charges). Compounds of the latter type that have two different ligands were called multicomponent complexes [Ishchenko and Shapovalov, 2004; Babko, 1955].

T.S. West and A. K. Babko's schools have immensely contributed towards the development of a variety of photometric techniques based on ternary complexes. West has classified ternary complexes into two major categories, these being the coordination-unsaturated type and ion-association type [West, 1967; Babko, 1955; 1965; 1968]. Babko formulated the conditions for the formation of triple complexes and suggested a classification that initially encompassed complexes of four types: 1) complexes having one ion of a metal, one electronegative ligand and an organic base; 2) complexes having one ion of a metal and two different electronegative ligands; 3) heteropolyacids; and 4) complexes made up of two different ions of metals and one electronegative ligand. However, due to the electrostatic interaction the second ligand Z^{v+} may attach to the coordinately saturated binary complex:
$\mathrm{MYn}^{\mu-}$: $\mathrm{MY}n^{\mu-} + \mathrm{Z}^{v+} \rightleftarrows [\mathrm{MY}n]^{\mu-}\mathrm{Z}^{v+}$. Compounds of precisely this type were considered as ion associates [Ishchenko and Shapovalov, 2004]. The unequivalency of

the ligands relative to the central inorganic ion is evident: the first ligand is attached due to the covalent bonds and the second, by coulombic forces.

This kind of compound, along with two-component and three-component complexes [Belcher, 1973; Beletskaya, 1975], was investigated in detail, especially within the framework of the method of liquid-liquid extraction [Blyum, 1970], in connection with the condition of electrical neutrality of the compound extracted, precisely to which the ion associates corresponds. It was assumed that in such systems the ion associate did not contain solvent molecules [Korenman, 1970; Ishchenko and Shapovalov, 2004]. Intensive study of the analytical systems based on ion associates in a chemical analysis was due to one fundamental fact: the presence of a dye in the associate guaranteed a several fold increase in the sensitivity in comparison with the method that uses extraction of a binary complex and in a number of cases preservation of the high selectivity of analytical determinations of metals [Babko, 1968; Belcher, 1973; Rao *et al.* 1998] (copper [Baily ,1966], iron [Vydra and Pribil, 1959; Knizek and Musilova, 1968; Hulànicki and Nieniewska, 1974], mercury [Ramakrishna *et al.* 1976; Mathew *et al.* 1992; 1995], zinc [Rao and Ramakrishna, 1980], cadmium [Rao and Ramakrishna, 1982; Kartikeyan *et al.* 1993], lead [Somasekhara Reddy *et al.* 1997], Molybdenum [Haddad *et al.* 1975; Rao and Ramakrishna, 1980; Filik *et al.* 2004; silver [Koh and Katon, 1979; Fujita *et al.* 1993], rare earth metals [Bhagavathy *et al.* 1993a, 1993b; Vijayalekshmy *et al.* 2003], noble metals [Jaya *et al.* 1983; 1984,Ramolingom Pillai *et al.*1997], actinides [Ramakrishna and Murthy, 1980] and many others).The associates include different classes of dyes, but preference was given to the most intensely colored (or most luminescing) ones, among which there were oxyand aminoxanthenes, sulfophthaleins, halogen derivatives of fluoresceine, cyanins, and a number of azo dyes [Rao *et al.* 1998].

Generalization of numerous works of this kind has led to the conclusion that metal-containing ion associates are compounds whose composition can be represented by the general formulas $[\mathrm{MR}n]\mathrm{R}m'$, $(\mathrm{AH})m[\mathrm{MR}n]$ or $[\mathrm{MA}m]\mathrm{M'R}n$, where M and M′ are the ions of metals, A stands for the organic base, and R or R′ are the electronegative ligands [Pilipenko and Tananaiko, 1983]. Among the basic characteristic features of the ion associates we may single out the following: (1) in all cases, metal enters into the composition of the complex cation or anion; (2) the counterion can be represented by an acid residue, organic base (amine), dye, onium cation, or a complex cation, of another metal; (3) an ion associate is an analog of an incompletely dissociated salt in solutions, an intermediate compound between simple salts and complex (coordination) compounds; (4) formation of an associate presupposes electrostatic interaction between oppositely charged ions, due to which they get closer, with the formation of a compound that is distinguished by considerably weaker interaction forces than in inner-sphere binary complexes. In the most generalized sense — to denote an entity of particles consisting of both identical and different molecules — the associates are defined as a "group of molecules of a liquid phase that has an internal (translational, orientational) structure owing to its origin to the formation of intermolecular bonds, the lifetime of which is at least an order of magnitude higher than the period of intermolecular vibrations" [Ishchenko and Shapovalov, 2004]. At present, the study of supermolecular self-organization of fluids has made it possible to reveal, in solutions, labile supermolecular associates (aggregates) having nanodimensions and consisting of dimers to dozens of molecules (alcohols, amides, etc.) [Durov, 1998; Shilov *et al.* 1999] can exist. Absorption and emission spectroscopy are the techniques most widely used and yield most information about aggregation due to observation of spectral shifts, Beer's law deviations or fluorescent quenching at high concentrations. Aggregates of ion pair (dye cation/anion- specific counter ion) entities can show interesting spectral manifestations due to different orientation of the ion pair aggregates as given below [Oakes and Dixon, 2004].

- parallel dimers (H-aggregates): the transition to lower energy excited state is forbidden, so the spectrum consists of a single blue-shifted broad band with respect to monomer
- head-to-tail dimers (J-aggregates): in this case, the transition to the higher energy excited state is forbidden, so the spectrum consists of a single red-shifted broad band with respect to monomer
- dimers having intermediate geometries: both transitions are partially allowed, and band splitting are observed so that the angle between transition dipoles can be calculated from the oscillator strengths of component bands.

The interest in this phenomenon has recently increased due to the advent of new types of machinery, perfection of technologies and creation of devices. The association of organic dyes exerts a substantial influence on their photophysical and photochemical properties that determine the application of dyes as converters of light energy in laser technology [Ishchenko, 1994], photographic industry [Shapiro, 2000], solar power engineering [Grin'ov *et al.* 2003], optoelectronics [Ishchenko, 2002] and biology and medicine [Valeur and Leray, 2000].

Basic non-chelating dyes enjoy high popularity in spectrophotometric analysis due to a high molecular absorption coefficient (about $1 \text{ X } 10^5 \text{ Lmol}^{-1}\text{cm}^{-1}$). They are able to form extractable ion-pairs with monovalent anionic complexes of metals leading to a variety of sensitive methods. Polyvalent anionic metal complexes also react with basic dyes, but the reaction products cannot be extracted by slightly polar solvents. Instead, the compounds formed accumulate during shaking on the phase boundary or on the wall of the separating funnel. The precipitate can be separated off and dissolved in a polar solvent, producing an intensely colored solution that forms the basis of flotation-spectrophotometric method of determination [Marczenko and Freiser, 1981].

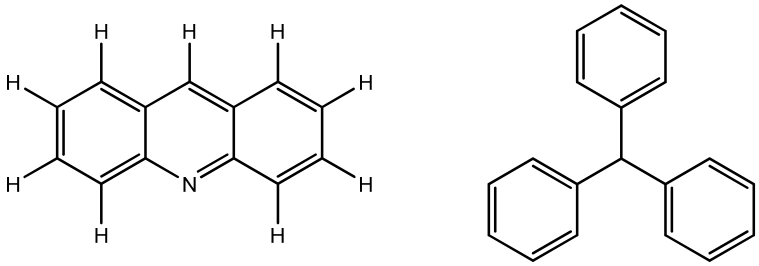
5.4. OPTICAL MOLECULAR PROBES IN AQUEOUS MEDIA

Ion association processes in water, with respect to the unique role of this solvent, are of great interest for physical organic chemistry and organic chemistry of electrolytes [Reichardt, 2003; Gordon, 1979] as well as for biochemistry and related sciences [Owen and Sultana, 1972; Reichardt, 2003]. In analytical chemistry, namely in extraction-photometric (fluorimetric) determinations, ion pairs formed in water are transferred to organic solvents; [Mchedlov-Petrossyan *et al.* 1995] association of oppositely charged ions in water are of importance for chromatography as well [Shtykov *et al.* 1999]. In the case of organic ion association in water, not only electrostatic interactions are of significance, but also hydrophobic ones, as well as Van der Waals attraction (including interactions between π- electronic systems) and hydrogen bond formation [Reichardt, 2003; Gordon, 1979; Kessler and Zaitsev, 1989]. Hence, much higher stability of associates in water can be expected for bulky organic ions as compared with those formed by small inorganic ones.

The effects of association of organic ions with opposite charges are rather versatile. The association of aryl and alkylsulfonates, alkylsulfates, alkylborates and other anions with cations such as alkyl and arylammonium, tetraphenylarsonium and tetraphenylphosphonium has been reported [Mukhayer and Davis, 1977; Savvin *et al.* 1991]. Other phenomenon, typical for aqueous dye solutions, is the association of dye ions with surfactant ions of opposite charge within the concentration range below the critical micelle concentration (cmc) of surfactant homomicelles, leading to step-by-step formation of poorly soluble salts and mixed dye-surfactant micelles [Buwalda and Engberts, 2001; Hadjianestis and Nikokavouras, 1993] . The interactions of such a kind are often accompanied by prominent changes in the visible spectra as, for instance, in the case of the association of hydroxyxanthene anions with quaternary ammonium drugs, [Shapovalov *et al.* 2005] or of the cationic dye neutral red with *n*-dodecylsulfate [Dutta and Bhat, 1993]. The limited stability of such systems sometimes requires the application of stabilizers, *e.g.* polyvinyl alcohol, which minimize the formation of deposits. Various mechanisms have been suggested to explain the forces holding dye ions together in solution. These include additive forces of a Van der Waals type, [Hadjianestis and Nikokavouras, 1993; Dutta and Bhat, 1993; Ishchenko *et al.* 1997] intermolecular hydrogen bonding, [Musha and Sugimoto, 1959; Gupta and Reed jr, 1970; Bishop, 1976] hydrogen bonding with the solvent [Buwalda and Engberts, 2001; Dutta and Bhat, 1993] and coordination with metal ions [Shapovalov *et al.* 2005].

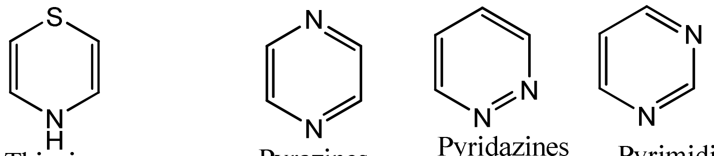
Colorimetry and spectrofluorimetry are essentially based on the formation of binary, ternary or multi-component complexes with most inorganic species [Haddad, 1977; Rao *et al.* 1998]. We can find several photometric methods of determination of trace metals from the compilation of existing methods done by Snell & Snell, Sandell and Onishi separately [Snell and Snell, 1959; Sandell,1959; Onishi 1989a; 1989b]. Fiegl's Spot tests for inorganic analysis remains a valuable companion for the qualitative detection of inorganics [Fiegl, 1962]. Of the modified methods for metal determination, those that do not require a preliminary solvent extraction step are especially advantageous due to the exclusion of environmentally hazardous organic solvents (such as chloroform and benzene) and to enhanced selectivity for the analyte (as even small anions as interferents may be extracted into organic solvents with large cationic reagents). The selectivity can be further enhanced by employing masking agents. The analysis in aqueous media employing ternary complexes are being preferred in green chemistry point of view and also the carcinogenity of most organic solvents

Basic or cationic dyes can be classified according to their structure, chromophoric group and heteroatoms into several classes, like acridines, thiazines, diazines, xanthenes, triarylmethanes, etc as shown in Scheme 5.1. These dyes have usually a positive charge that is delocalized over the whole Π-system of the molecule, with the largest electron densities placed on the nitrogen atoms. Xanthene molecules are well known for their remarkable photophysical properties. Therefore, they are frequently used as efficient laser dyes [Selwyn and Steinfeld, 1972; Wong and Schelly, 1974; Toptygin *et al.* 1997] and fluorescent probes [Ilich *et al.* 1996; Ajtai and Burghardt, 1995] attached to molecules of biological interest. The dyes which were studied in the present investigation are Rhodamine 6G and its pyronine analogue, Pyronine G, typical examples of the dyes of this class of compounds shown in Scheme 5.1. They were respectively chosen, the first one for its very frequent use as a laser dye and the second one as a simpler model of such dyes. Both of them contain the same chromophoric part, three flat conjugated cycles called chromophores which are responsible for the photophysical properties. The Rhodamine 6G carbethoxyphenyl group is nearly perpendicular to the chromophore and is therefore responsible for some steric constraints.


Acridines

Triaryl Methane

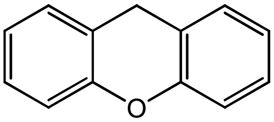
Diazines

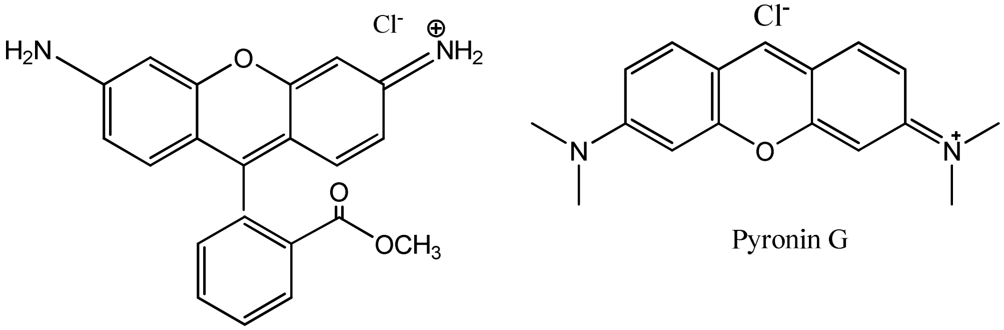

Thiazines

Pyrazines

Pyridazines

Pyrimidines


Xanthenes



Rhodamine 6G

Pyronin G

Scheme 5.1 Examples of Basic Molecular dyes and two representative examples of xanthene dyes: Rhodamine 6G and Pyronin G

Some of the properties that makes basic dyes interesting choice for their use as probes [Neumann and Tiera, 1993] are:

- Broad absorption bands in the visible and ultraviolet spectra, with quite large extinction coefficients (over 20,000 $M^{-1}cm^{-1}$). This permits the use of very small concentrations of the dyes, minimizing possible interactions of the system with the probe;
- High fluorescence quantum yields, usually about 20%;
- Very short fluorescent lifetimes (about a couple of nanoseconds), that assure that most properties of the systems will not be change during the measurements;
- Good sensibility to the environment, in the sense that emission and absorption spectra do, in general, depend on the environment where they are placed, changing the emission intensities (or extinction coefficients), as well as the shape of the spectra;
- Low reduction potentials, which allow the use of appropriate reducers to quench their excited states and emissions;
- Possibility of competition between uni- and bimolecular decay mechanisms
- Being the dyes essentially hydrophobic species, the presence of charge allows their localization in hydrophilic regions or interfaces;
- Well known dye aggregation processes in aqueous solutions and induced by microheterogeneous systems;
- Derivatives of the dyes are relatively easy to synthesize and bind to other molecules

An optical molecular probe may be assembled in aqueous media with a chromophoric receptor by means of intermolecular forces, including hydrogen bonding, π-π and hydrophobic interactions. Such an approach is attractive since not only it eliminates extra steps in synthesis, but it also allows for employment of combinatorial method: variation of dyes with the same ligand (or *vice versa*) may create libraries of ion-sensitive ensembles. The reaction is accompanied by significant changes in the fluorescence or absorption spectrum of the optical probe. Complex formation in a tri-component system metal cation (M^{n+})– anionic ligands (L) – dye cation (+) was expected to proceed in one of the two contrasting ways: competitive complexation of cationic dye and M^{n+} with L, which normally results in the dye displacement from its complex with anionic ligand by the metal ion, or assembly of a tri-component outer sphere complex ie. ionassociates. Significant improvement of the sensitivity and selectivity, lowering of the detection limits and decrease in the time taken for analysis can be achieved using the chemico-analytical properties of ion associates.

5.5 MULTI-ANALYTE SENSING

Over the past several years multi-analyte sensing has received increasing attention because of its applicability to clinical, environmental, chemical and biological analysis [Walt, 1992]. For example, measurements of pH, oxygen, carbon dioxide, calcium and electrolytes, glucose, antigens and antibodies, and specific DNA sequences are important for ascertaining the physical state of a patient. Detecting environmental pollutants (e.g. heavy metal ions and organics) [Oehme and Wolfbeis, 1997] is equally important as monitoring starting materials and end products to determine the efficiency of a particular industrial process. Recently, the rapid proliferation of multi-analyte sensing has prompted the development of miniaturized array sensors on solid supports. The major trends driving optical chemical sensor technology are miniaturization and multi-parameter functionality on a single platform (so-called multi-analyte sensing).

Table 5.2 Existing multi analyte sensing of heavy metals by spectral techniques

SL No.	Analytes	Technique	Dynamic Range (μg L^{-1})	Detection Limit (μg L^{-1})	References
1	Zn(II), Cd(II),Pb(II),Cu(II), Co(II) & Ni(II)	Solid Phase Spectrophotometry	5 -50 (Cd) 10-100 (Pb)	200 (Cd) 200 (Pb)	[Vukovic *et al.* 2007]
2	Cu(II), Pb(II) & Hg(II)	Derivative Spectrophotometry	25- 250 (Pb) 25- 250 (Hg)	20 (Pb) 20 (Hg)	[Mathew *et al.* 1995]
3	Cd(II) & Pb(II)	Flow injection Spectrophotometry (Chemometrics)	100– 1000 (Cd) 500 – 4000 (Pb)	270 (Cd) 120 (Pb)	[Castillo *et al.* 2001]
4	Cd(II), Pb(II) & Hg(II)	Spectrophotometry (Reflectance)	-----	340 (Cd) 620 (Pb) 600 (Hg)	[Czolk *et al.* 1992]
5	Cd(II), Pb(II) & Hg(II)	Spectrophotometry (Chemometrics)	396 (Cd) 9810 (Pb) 0-12,240 (Hg)	-	[Rouhollahi *et al.* 2007]
6	Zn(II), Cd(II) & Hg(II)	Reflectometry	0-2000 (Cd & Hg)	-	[Gomis and Garcia, 1990]
7	Pb(II) & Cd(II)	Spectrofluorimetry	0-250 (Pb) 0-150 (Cd)	0.5 (Pb & Cd)	[Gomis and Garcia, 1990]
8	Cd(II), Pb(II) & Hg(II)	Spectrofluorimetry	0-1000 (Cd),0-500 (Pb), 0-1100 (Hg)	4,3 & 6 (Cd, Pb & Hg)	[De la Riva *et al.* 2002]

Compared to the expensive multianalyte quantificiation techniques like ICP-MS, ICP-AES, TXRF and NAA spectrophotometric methods offer low cost, easy handling, portable and requires minimum manpower. Most of the spectrophotometric techniques for multimeasurand sensing are based on derivative spectrophotometry or chemometric approaches. A very few existing methods of multianalyte sensing of heavy metals by spectral techniques are given in Table 5.2. It can be noticed that techniques based on spectrophotometry are either derivative methods or based on reflectance or chemometric approaches which are tedious and time consuming. The need of the hour is to develop strategies for the real time sensing of analytes from a multicomponent sample at an affordable cost & with minimum manpower.

Recently Wallace [2009] has reviewed the molecular dyes used for the detection of biological and environmental heavy metals particularly Hg^{2+}, Cu^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, $Fe^{3+}$and Pd^{2+} while Lippard & coworkers [Nolan and Lippard, 2008] has provided a comprehensive account of recent progress in the design and application of optical sensors for mercury also covering complementary approaches that incorporate similar design principles and provide optical feedback, including Hg(II)-responsive biomolecules and materials. None of these methods are pertaining either to the multiplexed sensing of toxic heavy metals using off the shelf choromophores or ion associate interactions and there is a real need for such a strategy which can provide optical signatures of the multicomponent mixtures of heavy metals using economically viable method. Avenue for dual mode can significantly improve the sensitivity, selectivity and reliability of the technique.

The unique optical properties of xanthene dyes to form ion associates with anionic metal complexes and selectively controlling its behavior can be utilized for developing smart multiplexed sensing ensembles in the aqueous media. In the present investigation we have developed an analytical methodology for the sequential multianalyte sensing of toxic heavy metals from a single solution. A multicomponent sensing ensemble in solution based on unique optical characteristic exhibited by the spectroscopically ‘silent’ heavy metal pollutants viz. Cd(II), Pb(II) and Hg(II) iodides in presence of cationic xanthenes dyes is an interesting optical molecular probe. This can be achieved by the careful selection of the various complexing agents for the sequential switching of optical signals viz. absorbance or fluorescence which can provide multimodal transduction scheme.

Chapter 6

Ion Pair Based Optical Molecular Probes for Sequential Multimeasurand (heavy metal) Quantification

6.1 Abstract

A novel Ion Pair based optical molecular probe for the simultaneous visual detection and quantification of spectroscopically silent heavy metal toxins viz. cadmium, lead and mercury has been developed. This is based on the proposed sequential ligand - exchange Mechanism (SLE) of iodide from Pb - I^- *- Rhodamine 6G ion-associate with citrate (without affecting ion associates of Cd and Hg) and subsequently from Cd -* I^- *- Rhodamine 6G ion-associate with EDTA (without affecting Hg -* I^- *- Rhodamine 6G). Multi-measurand detection and quantification by colorimetry is possible as the individual toxins gives identical bathochromic shifts in aqueous solution, i.e. from 530 to 575 nm on formation of ternary ion associates in singular, binary and ternary mixtures. The visual detection provides a simple, quick and sensitive detection method in addition to quantification via spectrophotometry with Sandell sensitivities of 1.1, 15 and 2.5 μg* dm^{-2} *for cadmium, lead and mercury respectively. The developed procedure has been successfully tested for the analysis of environmental (cast alkali, lead acid battery and zinc manufacturing industry effluents) samples. Furthermore, the multi-measurand quantification of the above mentioned heavy metal toxins based on fluorescence quenching and use of Pyronine G as chromo-ionophore instead of Rhodamine 6G is also described.*

6.2 INTRODUCTION

Colorimetry continues to be widely popular in view of its simplicity, rapidity, precision, facile visual detection, common availability, low operational costs and possible field use. The chief limitation is selectivity, ie. discrimination of one measurand in presence of several analogous co-existing metal ions. This was circumvented via i) chemometric approaches [Vukovic *et al.* 2007; Raimundo and Narayanaswamy, 2003], ii) sequential extraction [Gomis and Garcia, 1990] and iii) by employing higher order derivative spectrophotometric techniques [Mathew *et al.* 1995]. Ternary complexes, wherein a central ion reacts with two different ligands, have aroused considerable interest among analytical chemists. Prof. West has classified ternary complexes into two major categories, these being the coordination- unsaturated type and ion-association type [West, 1967]. The co-ordination-unsaturated ternary complex results when a binary complex ("binary" here refers to a complex containing one type of central ion and one type of ligand, regardless of their stoichiometric ratio) has free co-ordination positions available on the central ion, allowing further reaction with a different ligand. Alternatively, if the binary complex is charged, then ion association with an ion of the opposite charge (usually a dyestuff) may occur. Colorimetric or spectrofluorimetric procedures based on association of $[ML_x]^{n-}$ complexes with cationic dyes in general involves extraction into immiscible organic solvent prior to 1976 [Rao *et al.* 1998; Onishi, 1989; Lobinski *et al.* 1992]. Prof. Ramakrishna's group have reported an aqueous procedure utilizing $[HgI_4]^{2-}$ (Rhodamine 6G)$_2$ color system by taking advantage of bathochromic shift that occurs in aqueous medium on formation of ternary ion associate [Ramakrishna *et al.* 1976]. This has triggered several aqueous phase based colorimetric procedures for variety of metal ions which have been successfully applied to diverse complex real samples by taking advantage of masking or sequestering agents to eliminate deleterious effects of coexisting interferent ions, if any [Rao *et al.* 1998].

A wide range of analytical methodologies based on ion associates of xanthene dyes and metal-ligand binary anionic complexes were developed from Prof. Ramakrishna's and Dr. Rao's groups. Significant contributions from these two groups has immensely widened the scope of ion associates of xanthene dyes from extractive spectrophotometric methods to design optical molecular probes in aqueous media enabling the methodologies applicable for real environmental sample analyses like soil samples, high purity zinc materials, osmiridium alloy samples etc. Among these methodologies the notable ones are ion associates based on metal iodides of heavy metals like mercury, zinc, cadmium, lead, noble metals, ziram etc for their selective and sensitive determination [Ramakrishna *et al.* 1976; Rao and Ramakrishna, 1980,1982; Jaya *et al.* 1983, 1984; Karthikeyan *et al.* 1993, Somasekhara Reddy *et al.* 1997, Mathew *et al.*1995]. Metal: ligand: Rhodamine 6G or Pyronin G ratio of 1:4:2 and 1:5:3 were established by Job's plot, mole ratio and equilibrium shift plot studies [Rao *et al.* 1998]. Optical molecular probes based on metal thiocyanates and xanthenes dyes were also been reported from Prof. Ramakrishna's group for zinc and molybdenum [Rao and Ramakrishna, 1980a, 1980b]. Xanthene dye based ion associate system for actinide group metals especially uranium(VI) using anthranilic acid-Rh 6G system has widened the scope of these optical probes [Ramakrishna and Murthy,1980].

The concept of sequential ligand exchange (SLE) was advantageously utilized (though not mentioned) to improve selectivity by addition of EDTA to lead - bromopyrogallol red binary complex several decades back [Belcher *et al.* 1965] and described recently by Balaji and Matsunaga during fluoride quantification [Balaji and Matsunaga, 2005]. To our knowledge, SLE concept has not been used and reported for successive determinations of binary or ternary analyte mixtures. In this chapter we present the findings pertaining to sequential detection and quantification of spectroscopically silent heavy metal toxins viz. cadmium, lead and mercury in industrial effluent samples by using [$(ML_4)^{2-}$ $(Rh6G)_2{}^{+}$] ion pair based optical molecular probes.

6.3 ANALYTICAL METHOD DEVELOPMENT

6.3.1 Absorption Spectra and Spectral Characteristics

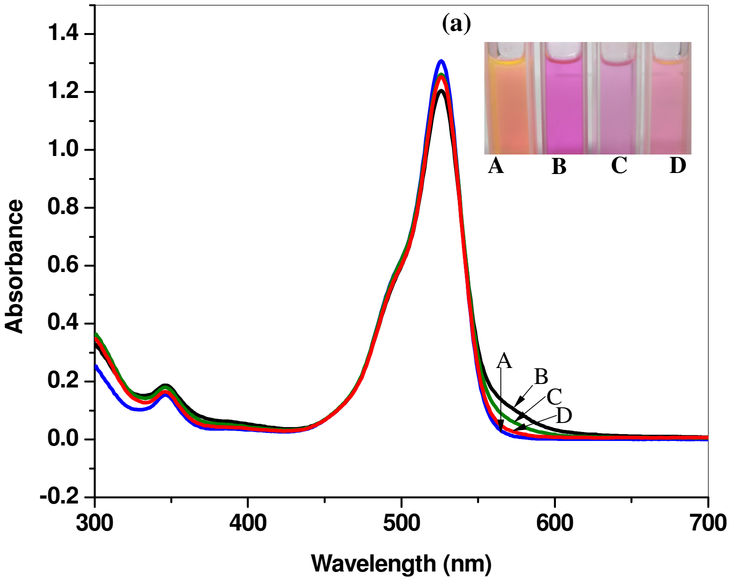


Figure 6.1a *Absorption spectra of the Rhodamine 6G (Curve A), MMS – iodide - Rhodamine 6G (Curve B), on addition of citrate (Curve C) and on further addition of EDTA (Curve D).* **A:** *1 mL of 1 mol* L^{-1} *of pH 5 hexamine buffer + 2.0 mL of* $2x10^{-4}$ *mol* L^{-1} *Rhodamine 6G + 2 mL of 5% KI + 1 mL of 1% gelatin in a total volume of 25 mL;* **B:** *as in A but with the addition of 4 mL of MMS solution;* **C:** *on addition of 1 mL of 0.4 mol* L^{-1} *citrate buffer to solution of B, and* **D:** *on further addition of 1 mL of 0.1 mol* L^{-1} *EDTA to solution of C; The photographs of solutions of A,B,C & D in quartz cell are shown in inset (a)*

Figure 6.1a shows the absorption spectra of 2mL of $2x10^{-4}$ mol L^{-1} Rhodamine 6G (Curve A) with 4 mL of MMS (Multimeasurand solution) (Pb(II), Cd(II) & Hg(II) (Curve B) in presence of 2 mL 5% KI and 1 mL of 1% gelatin, in a final solution of 25 mL. It is evident from the figure that the interaction of iodo- complexes of chosen heavy metal toxins with Rhodamine 6G proceeds with an identical bathochromic shift, the pink ternary complexes absorbing at 575 nm compared with 530 nm of orange red colored Rhodamine 6G dye, thus facilitating aqueous finish as the dye has negligible absorption at 575 nm. On sequential addition of 1 mL 0.4 mol L^{-1} of citrate buffer and 1 mL of 0.1mol L^{-1} EDTA (curves C&D respectively) the absorbance at 575 nm diminishes corresponding to Pb(II) and subsequently Cd(II).

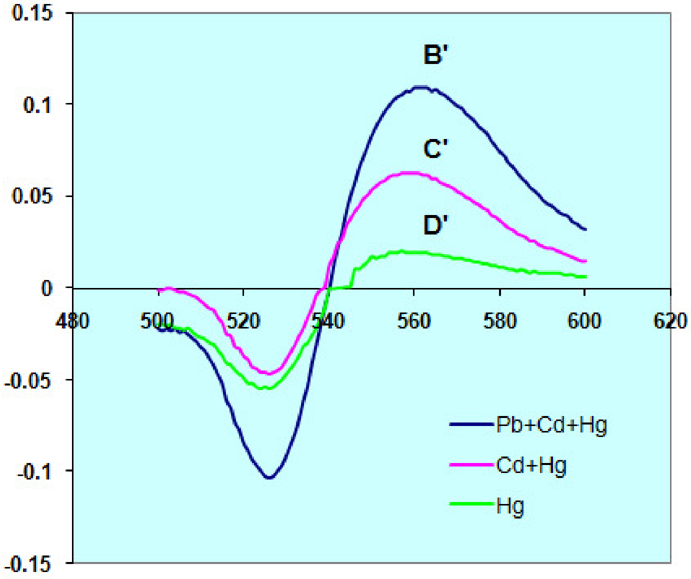


Figure 6.1b *Curves B', C' and D' → Absorption spectra of solutions of B, C and D measured against Solution A.*

The absorption spectra of solutions representing curves B, C and D measured against solution A (Blank) are depicted in curves B', C' and D' respectively. Masking of Pb(II) on the addition of citrate buffer weakens the intensity of the absorption signal of Pb(II), Cd(II) and Hg(II) mixture on curve B' at 575 nm resulting in curve C' where the signal is purely of Cd(II) and Hg(II). Further addition of EDTA masks signal due to Cd(II) and give rise to curve D' of lower intensity.

6.3.2 Optical Molecular Probe (OMP) Design

An aliquot of sample solution (upto 15 mL) and 1.0 mL of hexamine buffer were taken in a 25 mL beaker and pH was adjusted to 5.2 ± 0.2 using HNO_3 or NaOH. Potassium iodide (2.5 mL) and Rhodamine 6G (5 mL) followed by gelatin (1 mL) were added, transferred to 25 mL volumetric flask and made up with water. The absorbances were measured at 575 nm in 10 mm quartz cells against a reagent blank (A1). The solutions were transferred to 50 mL volumetric flasks and 1 mL of citrate buffer was added to sample as well as blank ie. total volume : 26 mL. The solutions were mixed and measured the absorbance (A2) against the corresponding reagent blank solution. Again, the absorbance (A3) was measured after the addition of 2 mL of EDTA to above blank and sample solution ie. total volume at this stage : 28 mL against the corresponding reagent blank. After measuring first and second stage absorbances ie. A1 & A2, the solutions in quartz cuvettes were transferred back to respective volumetric flasks in case of samples and blank as well. Calibration plots were constructed for (A1-A2), (A2-A3), and A3 Vs volume of MMS for Pb(II), Cd(II) and Hg(II) respectively. The heavy metal toxin content was arrived at by referring to the corresponding calibration graphs.

6.4 OPTIMISATION OF EXPERIMENTAL PARAMETERS

Sequential optimization of experimental parameters like buffer system, pH, amount of KI, Rhodamine 6G, gelatin etc at the λ_{max} of ion associates (575nm) in each stage has been done. Influence of each component has been critically evaluated.

6.4.1 Effect of medium

In the first stage of mixture analysis, it was found that the expected cumulative absorbances for the three component viz. Pb(II), Cd(II), Hg(II) mixture were comparatively less in acetate buffer. This may be attributed due to the higher stability constants of Pb for acetate (log $K_f = 8.2$) as compared to iodide (log $K_f = 4.47$) [Martell and Smith, 1982]. Also it was found that in the second stage when 2.5 ml of 0.8M citrate was added to mask Pb alone, Cd signal was also found to be masked. This was evident as the concentration of citrate was reduced to 2.5ml of 0.4M citrate, Cd signal masking was reduced. Hence we have changed the acetate medium with hexamine medium as it has got less interaction with the analytes. Also the concentration of citrate in the 2nd stage was optimized to avoid the masking of Cd along with that of Pb.

6.4.2 Effect of pH

As the formation of respective ion associate between the binary metal-iodide anionic complex and rhodamine 6G cation are pH dependent, sequential optimization of pH in each stage was undertaken. The net absorbance signal at each stage is strongly dependent on the pH as shown in Fig 6.2. At pHs lower than 4, the liberation of I_3^- interferes the formation of ion associate between binary $[MI_4]^{2-}$ complex and rhodamine 6G resulting in high blank absorbances which considerably shadows the absorbance change due to the formation of $[(MI_4)^{2-}\ (Rh6G)_2{}^+]$ ion pair based molecular probes. The enhanced blank signal is due to the formation of ion associate of Rhodamine 6G and I_3^-. At higher pHs above 6, the formation of metal hydroxides competes with binary $[MI_4]^{2-}$ complexes, thereby reduces the analytical signal drastically. The formation of ion-associate as well as its flexible nature was maintained in the pH range 5 – 5.5 as shown in Fig 6.2 and we have selected an optimum pH of 5.2 throughout our studies.

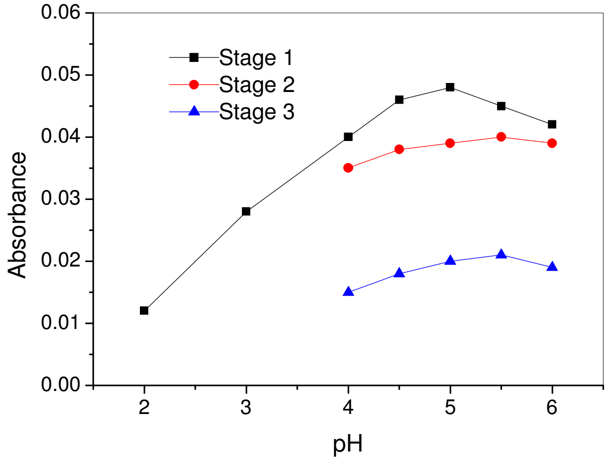


Figure 6.2 *Effect of pH*

6.4.3 Effect of KI concentration

The effect of iodide concentration was studied in all 3 stages. The largest difference in absorbance was obtained when 2.5 ml of 10% KI was added to the sample mixture which corresponds to an overall concentration of 60mM of iodide (ie ~2.5 x 10^4 fold compared to heavy metal ion) in the final determining solution as shown in Figure 6.3.

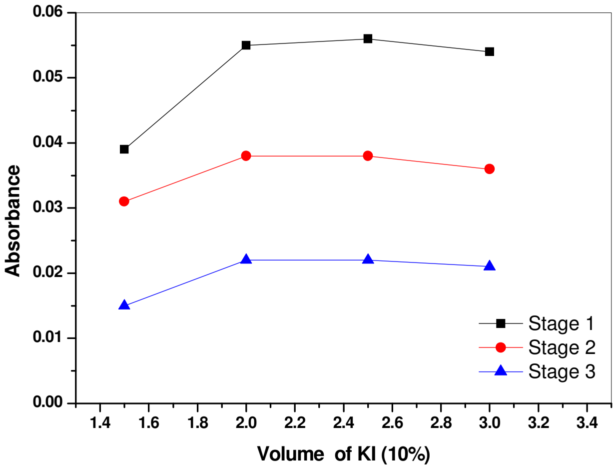


Figure 6.3 *Effect of iodide concentration*

This indicates that presence of a good excess of I^- anion is essential for the formation of metal iodide as they are at very low ppb levels, which can drive the equilibrium to forward direction resulting in the formation of binary $[ML_4]^{2-}$ anionic complexes which then forms ion associates with cationic xanthene dyes. KI solution has to be prepared freshly in triple distilled water, to avoid dissolved O_2, as it can oxidize iodide to I_2 to form I_3^- which interacts better with Rhodamine 6G analogous to $[ML_4]^{2-}$ culminating in variable blank values.

6.4.4 Effect of amount of Rhodamine 6G

The influence of amount of Rhodamine 6G on the formation of ternary ion associates with metal iodides Pb(II), Cd(II) & Hg(II) has been studied in each stage (shown in Figure 6.4) and has arrived at the conclusion that as low as 5ml of 0.01% of Rhodamine 6G corresponds to 4.2 x 10^{-5}M is required for the optimum absorbance change in each stage and the system is flexible enough for the sequential ligand exchange.

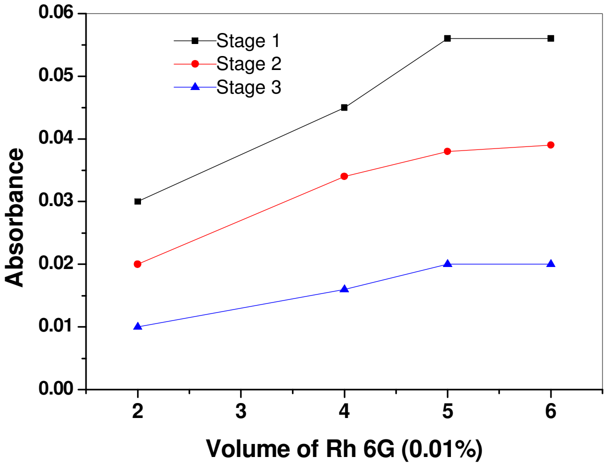


Figure 6.4 *Effect of Rhodamine 6G Concentration*

The spectral characteristics displayed by xanthene dyes have been attributed to the rigidity of their molecular structure arising from the oxygen bridges between the benzene rings [Haddad, 1977]. Some key characteristics of this dye class include low pH sensitivity and tunable spectral properties especially the N-alkyl derivatives. The molar absorptivity of the order of $\varepsilon = 1.16 \times 10^5$ l mol^{-1} cm^{-1} for rhodamine 6G in aqueous solution at λmax = 530 nm is due to the conjugated structure of the dye, R^+, by means of which the dye can form ion-association complexes with anions through the quaternary ammonium group (the dye does not have chelating functional groups).

6.4.5 Effect of amount of gelatin

The effect of amount of gelatin concentration on analytical signal at all 3 stages was studied and the results obtained are shown in Fig 6.5. As low as 1ml of 1% gelatin were required for obtaining constant and maximum absorbance. The order of addition of reagents indicates that gelatin has to be added after the formation of ternary ion association complex. The addition of gelatin prior to Rh 6G and KI lowers the analytical signals. This indicates prior addition of gelatin prevents certain degree of aggregation of ion pair. This has been attributed to micellar effect which enhances absorbance in addition to bathochromic shift [Filik *et al.* 2004]. Gelatin is a protein produced by partial hydrolysis of collagen extracted from the boiled bones, connective tissues, organs and some intestines of animals such as domesticated cattle, pigs and horses [Kozlov, 1983]. The natural molecular bonds between individual collagen strands are broken down into a form that rearranges more easily. Gelatin melts to a liquid when heated and solidifies when cooled again. Being a protein, gelatin is composed of a unique sequence of amino acids.

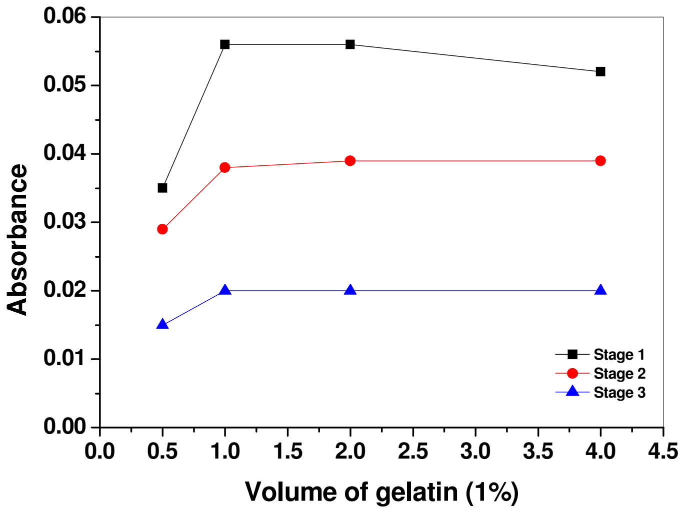


Figure 6.5 *Effect of gelatin concentration*

Characteristic features of gelatin are the high content of the amino acids glycine, proline and hydroxyproline. Structurally, gelatin molecules contain repeating sequences of glycine-X-Y triplets, where X and Y are frequently proline and hydroxyproline amino acids. Gelatin forms a solution of high viscosity in water and is capable of forming hydrogen bonds using the polar groups of the gelatin, such as peptide, carbonyl, and guanidine groups [Yi *et al.* 2006] with the dye carbonyl or amine functional groups. Moreover, gelatin is a 'stabilizer' type of emulsifying agent which increases the viscosity of the continuous phase and thus reduces the mobility of droplets in order to prevent them from coalescing [Behrend *et al.* 2000]. Hence, gelatin was added for promoting the formation of J-aggregates of the dye-metal iodide ion associates as well as to stabilize optical molecular probes of the ternary ion-association complex formed from $(Rh6G)_2^{+}$–$[ML_4]^{2-}$ to a certain extent thereby delaying the further aggregation leading to precipitation [Gorner *et al.* 2006]. This precludes the need for environmentally unfavourable solvent extraction step.

6.4.6 Stability

The test for colour stability showed that the absorbance measurements (at 575 nm) should be performed within 30 min (after final mixing of reagents) in the first stage and the stability of the color system remains for 15 min each for second and third stage. Also it was noticed that gelatin plays a major role in stabilizing the color system. The color system was found to be unstable in the absence of gelatin as the ion associate tends to precipitate out thereby affecting the spectral measurements. Figure 6.6 shows the stability pattern of the color system in three stages.

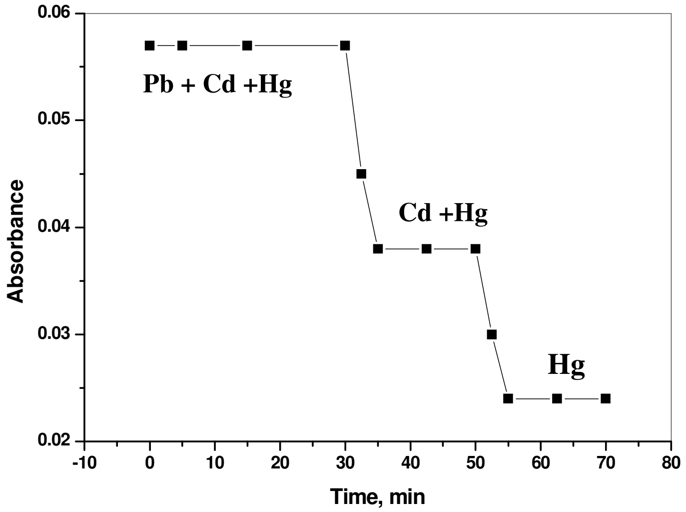


Figure 6.6 *Stability of the color system in 3 stages*

Optimal amounts selected eventually after optimization of various experimental variables viz pH, potassium iodide, Rhodamine 6G, gelatin, λ_{max} and stability are summarized in Table 6.1

Table 6.1 *Effect of different parameters during multi-analyte determination of admixtures of cadmium, lead and mercury by colorimetry (0.5 mol L^{-1} hexamine buffer (pH 5.0), 2.5 mL of 10% KI, 5.0 mL of 0.01% Rhodamine 6G and 1 mL of 1% gelatin (except when varied) total volume of 25 mL.*

Parameter	Optimal range			Selected Condition
	I stage	II stage	III stage	
pH	4.0 – 5.5	5.0 – 5.5	5.0 – 5.5	5.2 ± 0.2
KI (10%) mL	2.0 – 3.0	2.0 – 2.5	2.0 – 2.5	2.5
Rhodamine 6G (0.01%), mL	≥ 5	≥ 5	≤ 5	5.0
Gelatin(%), mL	≥ 1	≥ 1	≥ 1	1.0
Stability (h)	30 min	15 min	15 min	15 min
λmax, nm	575	575	575	575

6.5 SEQUENTIAL MULTIMEASURAND QUANTIFICATION OF CADMIUM, LEAD AND MERCURY

Under selected experimental conditions, the color systems obeys Beer's Law in the range 0-100, 0-1000 and 0-200 μgL^{-1} of cadmium, lead and mercury in admixtures, with sandell sensitivities of 1.1, 15 and 2.5 μgdm^{-2} respectively on measuring absorbance at 575 nm (Figure.6.7). The resulting linear regression equations are as shown in equations (1) – (3):

$$A_{Cd} = 7.90 \times 10^{-4} C_{Cd} + 1.75 \times 10^{-4} \text{ ---------------- } (1)$$

$$A_{Pb} = 6.07 \times 10^{-5} C_{Pb} + 3.05 \times 10^{-4} \text{ ---------------- } (2)$$

$$A_{Hg} = 6.75 \times 10^{-4} C_{Hg} - 7.63 \times 10^{-3} \text{ ---------------- } (3)$$

The corresponding correlation coefficients are 0.9979, 0.9984, 0.9915 for Cadmium, Lead and Mercury respectively, where A_{Cd}, A_{Pb}, A_{Hg} are A_2 - A_3 (for Cd), A_1 - A_2 (for Pb) and A_3 (for Hg) and C is the concentration of particular metal ion in µg L^{-1}. A_1, A_2, A_3 are absorbances measured at stages I, II and III respectively. All above statistical calculations are based on the average of duplicate measurements. The detection limits of the present method are found to be 6, 15 and 60 µg L^{-1} for Cd, Hg and Pb respectively. Furthermore, duplicate determinations on synthetic multi-measurand solution (MMS-II) that contained (25 + 250 + 50) µg L^{-1} of Cd, Pb and Hg respectively gave a mean recovery of 97.6 %, 100.5%, and 102.1% with RSD values of 2.13%, 2.15% and 1.74% respectively.

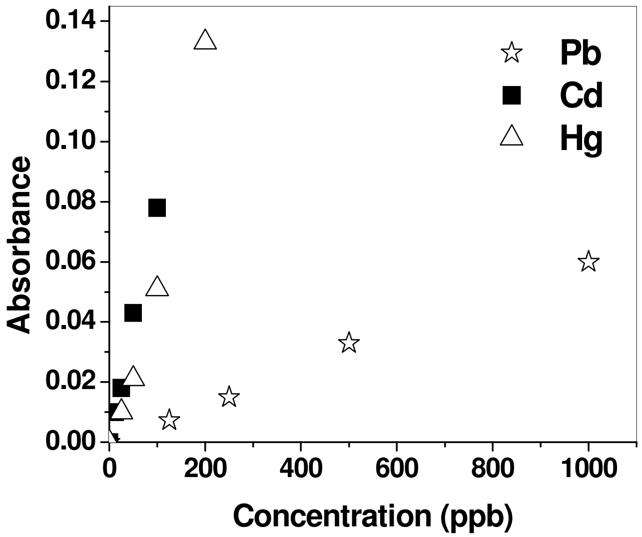


Figure 6.7 *Calibration plots of the sequentially determined Cd, Pb, & Hg in the concentration range 0-100, 0-1000 and 0-200 µgL^{-1} respectively*

6.6 STUDIES WITH PYRONINE G AS CHROMO-IONOPHORE

Prof. Ramakrishna's and Rao's group [Rao and Ramakrishna, 1982; Mathew *et al.* 1992] have described spectrophotometric procedures for cadmium and mercury respectively using Pyronine G as chromo-ionophore and demonstrated similar analytical characteristics as that with corresponding metal - I^- - Rhodamine 6G systems. Hence, experiments were conducted on the lines mentioned above for sequential multi-measurand determination of cadmium, lead and mercury with Pyronine G instead of Rhodamine 6G. The linear equations with regression are as shown in equations (4) – (6):

$$A^I_{Cd} = 6.16 \times 10^{-4} C_{Cd} + 6.09 \times 10^{-4} \text{------------------} (4)$$

$$A^I_{Pb} = 5.70 \times 10^{-5} C_{Pb} + 9.74 \times 10^{-3} \text{-----------------} (5)$$

$$A^I_{Hg} = 8.61 \times 10^{-4} C_{Hg} - 3.07 \times 10^{-3} \text{----------------} (6)$$

with corresponding correlation coefficients of 0.9999, 0.9918, 0.9979 where A^I_{Cd}, A^I_{Pb} and A^I_{Hg} are the absorbances (A_2^I- A_3^I) for Cd, (A_1^I – A_2^I) for Pb and A_3^I for Hg and C is the concentration. A_1^I, A_2^I and A_3^I are measured absorbances at stages I, II and III respectively.

6.7 FACILE VISUAL DETECTION OF HEAVY METAL TOXINS

The applicability of the present chemo-sensor for visual detection in form of spot tests and thin layer or paper chromatography plates or strips respectively, was tested. The color change from orange red to pink was clearly noticed visually in the presence of cadmium, lead, and mercury. Diminishing of the above pink color after addition of citrate indicates the presence of lead, further decrease in pink color after further addition of EDTA indicates the presence of cadmium and finally the remaining pink color is solely due to mercury (See inset (a) of Fig.6.1). The color changes are instantaneous even at room temperature and remain unaffected in all 3 stages for a period of 15 min.

6.8 EFFECT OF COEXISTING IONS

The interfering effect of 20 mg L^{-1} of selected heavy metal ions, which are likely to coexist in real samples on the determination of a mixture of Cd, Pb and Hg (MMS-II), was studied. The results indicate that Zn (II), Co (II), Ni (II), Mn (II) and Fe (III) have no deleterious effect in all the 3 stages, thus facilitating selective and sequential multi-measurand quantification of cadmium, lead and mercury in complex mixtures. 40 mg L^{-1} of Ca (II) & Mg (II) was found to have no deleterious effect on the sensing at all the three stages. The corresponding tolerance limits are shown in Table 6.2.

Table 6.2 *Tolerance limits of interferents in the determination of multimeasurand sample, MMS II (25 +50 + 250 μgL^{-1}) of Cd, Hg and Pb respectively*

Interferents	Tolerence limits (folds)		
	Pb(II)	Cd(II)	Hg(II)
Zn(II), Co(II), Ni(II), Mn(II), Fe(III)	80	800	400
Ca(II), Mg(II)	160	1600	800

6.9 FLUORIMETRIC STUDIES

Considering that all the basic dyes are strongly colored, it is not surprising that a spectrophotometric method was often developed simultaneously with the fluorimetric method. As a general rule, however, the fluorimetric method has proved more sensitive than the equivalent spectrophotometric method by at least a factor of two [Haddad, 1977]. This has enabled the same method to be used for a wide range of concentrations of analyte, with a spectrophotometric finish for higher concentrations and a fluorimetric finish for lower concentrations; greater versatility of the analytical method was therefore achieved. Thus, on addition of Pb(II) ,Cd(II) or Hg(II) either individually or in mixture, the dye is self-assembled resulting in quenching of fluorescence intensity of Rhodamine 6G at 556 nm on formation of J-aggregates of ternary ion associates. The emission intensity is drastically quenched due to diminishing of un-complexed dye entities. Later on, the fluorescence intensity is partly turned 'ON' with the addition of citrate and EDTA sequentially as more and more free dye is released by competitive ligand binding as shown in Figure.6.8. This enabled sequential fluorimetric determination of Pb(II), Cd(II) & Hg(II) in admixtures with detection limits of 4.1, 0.5 and 1.6 μg L^{-1} respectively.

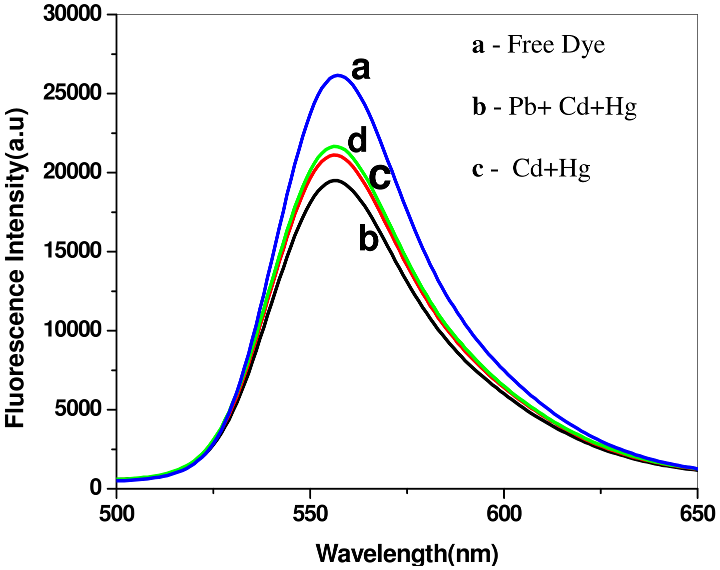


Figure 6. 8 *Fluorescence spectra of the MMS – iodide - Rhodamine 6G complex at pH 5 hexamine buffer Curve (a) : 5.0 mL of 4x10^{-4} molL^{-1} Rhodamine 6G + 2 mL of 5% KI + 1 mL of 1% gelatin; Curve (b): as in (a) but with the addition of MMS (2 μ molL^{-1} Pb, 0.4 μ molL^{-1} Cd, 0.8 μ molL^{-1}Hg) solution. Curve (c): on addition of 1mL of 0.4 molL^{-1} citrate buffer to solution of Curve (b), and Curve (d) : on further addition of 1 mL of 0.1 molL^{-1} EDTA to solution of Curve (c) in a final volume of 25mL.*

6.10 MECHANISM OF SEQUENTIAL LIGAND EXCHANGE

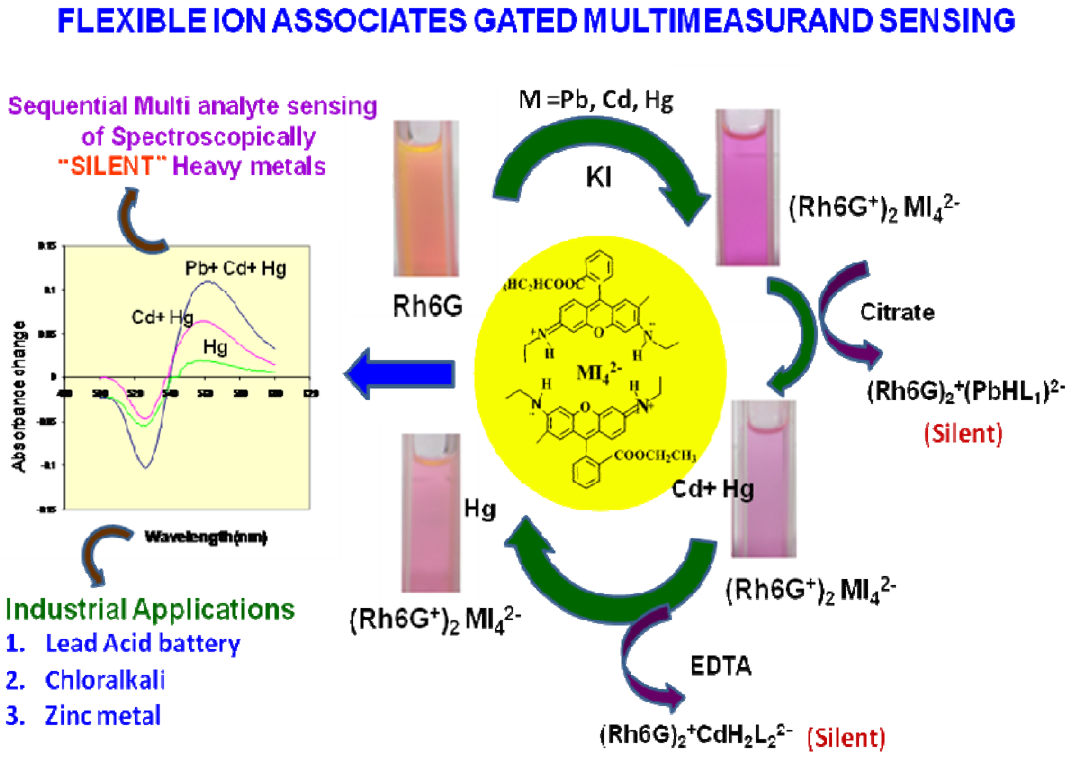
A unique feature exhibited by the 3 heavy metal toxins resulting in identical bathochromic shift i.e. 575 nm of pink ternary ion associates to that of 530 nm of orange red Rhodamine 6G or Pyronine dye has paved the way for sequential naked eye detection of admixtures of spectroscopically silent heavy metal toxins viz., Pb(II), Cd(II) & Hg(II) using off-the-shelf chromo-ionophore without the need for dedicated synthesis protocols. Belcher *et al.* [1965] have employed EDTA as masking agent to enhance the selectivity of niobium-bromopyrogallol red (BPR) color reaction. The EDTA replaces one BPR molecules of Nb - $(\mathrm{BPR})_3$ complex. Such ligand transfer has been advantageously utilized by Balaji and Matsunaga [2005] for naked eye detection of fluoride using Zr(IV) - EDTA - pyrocatechol violet color system. A similar mechanism can be thought of in the present case (See Scheme.6.1 for proposed sequential ligand exchange mechanism). Sequential ligand transfer of iodide with citrate (to mask lead) and EDTA (to mask cadmium) can be readily understood from the reported stability constant data of Pb(II), Cd(II) and Hg(II) with iodide, citrate and EDTA (See Table 6.3) [Martell and Smith, 1982]. The flexibility of the ion associate enabled the sequential ligand exchange mechanism in the multimeasurand mixture a reality.

The addition of citrate or EDTA also form anionic complexes with lead and cadmium respectively like iodide and form ternary ion associates but at higher concentrations of metal ions as $[\mathrm{M}–(\mathrm{HL}_1)_2^-]^{2-}$ and $[\mathrm{M}(\mathrm{H}_2(\mathrm{L}^-)_2]^{2-}$ binary anionic complexes in pH 5 solutions. Evidence to existence of $[\mathrm{M}\ (\mathrm{HL}_1)_2]^{2-}$-$\mathrm{R}_2^+$ and $[\mathrm{M}\ (\mathrm{H}_2\mathrm{L}_2)_2]^{2-}$-$\mathrm{R}_2^+$ ternary ion associate was proved by extracting into benzene with 200 μgL^{-1} of each of respective metal ion. In addition, under the present experimental conditions where the metal ion concentrations are in ppb levels, binary lead citrate and Cd-EDTA complexes may also be formed. In spite of subtle differences in stability constants (K_4) of Pb(II) or Cd(II) or Hg(II), all ternary ion associates resulted in identical bathochromic shifts as clearly seen from Fig.6.1a and also as reported elsewhere for cadmium [Kartikeyan *et al.* 1993], lead [Somasekhara Reddy *et al.* 1997] and mercury [Ramakrishna *et al.* 1976]. This enabled total metal ion i.e. [Pb(II) + Cd(II) + Hg(II)] content in stage I. The addition of citrate, selectively exchanges iodide ligand of $[PbI_4]^{2\,-}$(R_2) with citrate (L_1) as the stability constant [Pb (HL_1)] is higher than [Pb $I_4]^{2-}$ while the stability constant $[CdI_4]^{2-}$ and $[HgI_4]^{2-}$ complexes are higher than their corresponding citrate complexes (See Table 6. 3). The measurement of absorbances at this stage (Stage II) gives the concentration of [Cd(II) + Hg(II)].

Table 6.3 *Cumulative formation constants of metal – ligand complexes*

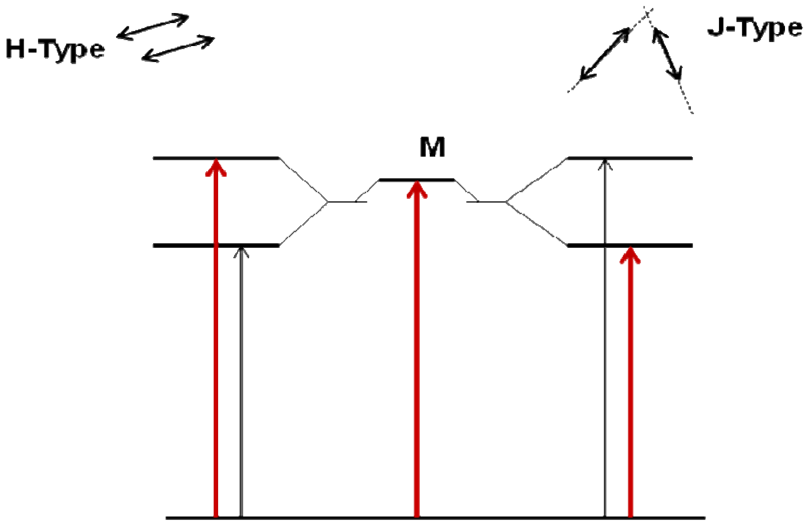
Ligand	Formation constant (log K_4) [Martell and Smith, 1982]		
	Cd	Pb	Hg
Iodide	5.41	4.47	29.83
Citrate	3.98	6.50	10.90
EDTA	11.00	18.30	21.80

The subsequent addition of EDTA replaces iodide ligand of $[CdI_4^{-}]^{2-}(R)_2^{+}$ as the stability constant of $[Cd(H_2L_2)]$ is higher than $[CdI_4]^{2-}$ while the stability constant of $[HgI_4]^{2-}$ is higher than $[Hg(H_2L_2)_2]^{2-}$(See Table 6.3). The measurement of absorbance at this stage (Stage III) essentially corresponds to that of mercury. The difference in absorbances of Stage II from Stage III corresponds to cadmium while that of Stage I and Stage II is due to lead respectively and that at stage III is due to Hg (II) alone. Thus, a sequential ligand exchange of iodide with citrate and EDTA enabled simultaneous multi-measurand quantification of heavy metal toxins viz Pb(II), Cd(II),and Hg(II).



Scheme 6.1 *Schematic representation of proposed sequential Ligand Exchange*

Figure 6.1a shows the absorption spectra of the ion associate is red shifted from 530 nm to 575 nm. These spectral changes are attributed to the aggregation of dyes which is promoted by the metal iodides due to increased hydrophobicity of the ion pair in comparison with individual ions. The aggregates are probably consisted of not only double ion pair but charged ion pair formed by the adsorption of the Rh6G$^+$ ion to the ion pair [Lee *et al.* 1989]. As the dissociation constant of dimer of the dye dimer is 5.9 x 10^{-4}, essentially all the dye exists as the monomeric form in the absence of metal iodides [Selwyn and Steinfeld, 1972]. From figure 6.1a it can be seen that ML_4^{2-} apparently promotes the dimer of the J band which is evident from the bathochromic shift to 575nm. Moreover coloumbic repulsion can be minimized if the charged amino groups in $Rh6G^+$ lie along the far edges as possible. These changes can be qualitatively explained by exciton theory based on interactions between the dipole moments of the monomeric units [Muto, 1976]. According to the theory a dye monomer peak may split into a higher energy band, H band, and a lower one, J band, as a result from the interaction between adjacent dye cations in the aggregation of ion pair. The dipole-dipole interactions result in a splitting of the monomer excitation which depends on the geometry of the aggregates. The geometry with the head-to-tail transition dipoles leads to a decrease in energy, and the geometry with the parallel transition dipoles leads to an increase in energy (see the exciton energy diagram in Scheme 6.2).



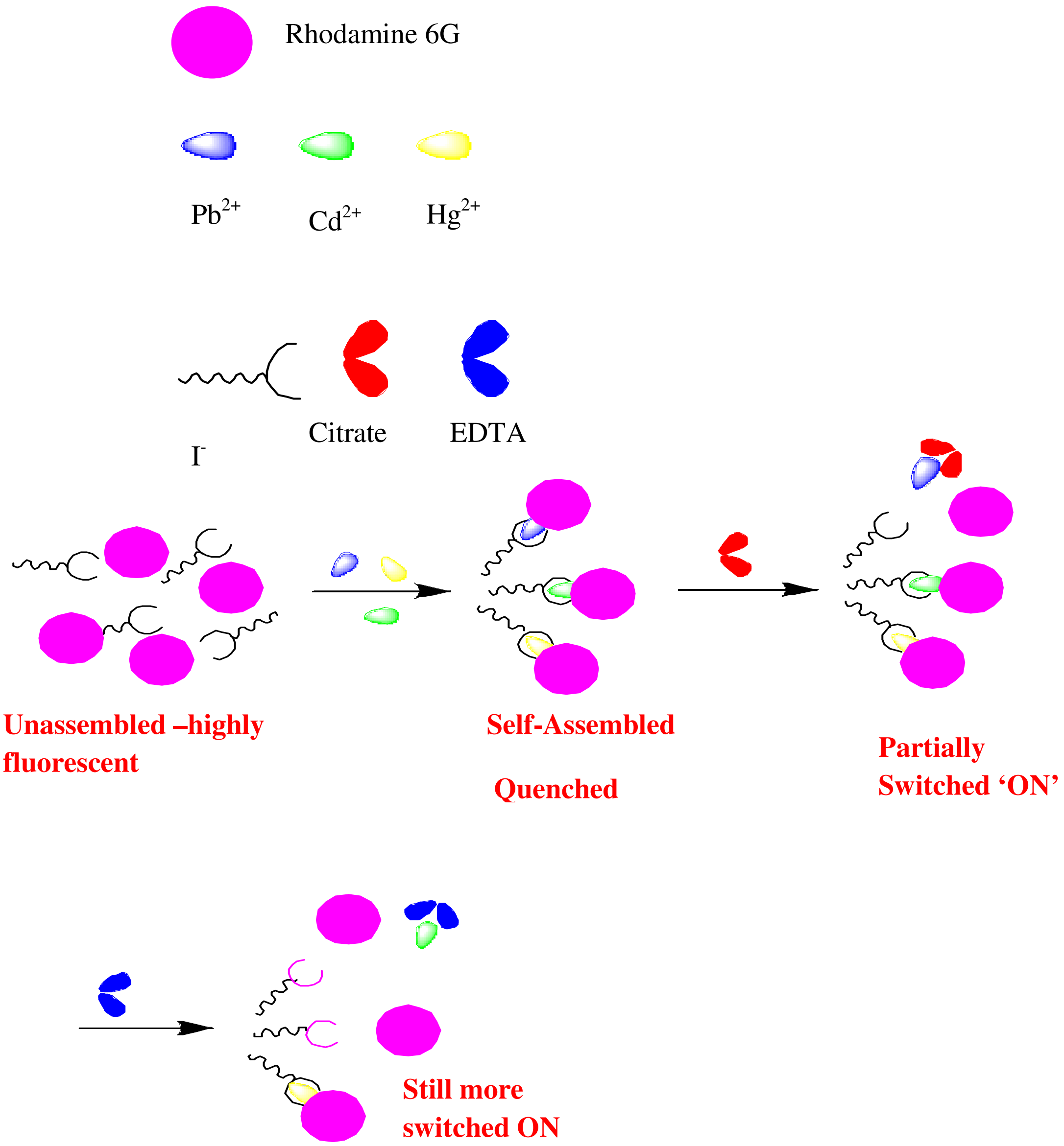
Scheme: 6.2 *Exciton splitting diagram of the electronic states for R6G monomer and two types (H or J) of dimers, according to the arrangement of the dipole moments. M represents the R6G monomer excitation. H-type and J-type splitting results in an increase or decrease in the excitation energy. The brown arrow represents the strongest transition.*

Therefore, the absorption of J-type dimers is predicted to red. Originally discovered by Jelley and Scheibe, [Jelly, 1936; Eisfeld and Briggs, 2007] J-aggregation was found to occur in neat aqueous solution or in mixtures with methanol, in the presence of metal ions, surfactants, in Langmuir-Blodgett films, and at the phospholipid vesicle surface, as well as in the presence of macromolecules such as polyelectrolytes, DNA, polyvinylsulfonate, and gelatin [Gorner *et al.* 2006; Wang *et al.* 2000].

Electrostatic and hydrophobic interactions are major driving forces in J-aggregation. The pH is a decisive factor which governs the rate and the yield of J-aggregates. J-aggregation is a cooperative process, and the number of the binding sites is one of the major factors controlling the J-aggregate size [Slavnova *et al.* 2007]. Gelatin is a heterogeneous mixture of polypeptides and is known to play an important role in the production of photographic emulsions and to have an enhancing influence on J-aggregation. Since gelatin comprises variously charged species at any pH, it behaves like an acid or a base with an isoelectric point around pH 5 and can be considered to be a polyelectrolyte [Dickinson, 1949; Gorner *et al.* 2006]. Thus the J-aggregates of xanthene dyes in presence of metal iodide and gelatin represent a nanostructured selfassembled supramolecular system. The formation of J-aggregates was considered to be a reversible reaction and was rationalized in terms of autocatalysis. Thus upon introducing suitable masking agents ie citrate, EDTA etc, specific metals are driven away from the flexible ion associates, and a concomitant decrease in absorbance of J-aggregates is observed.

Furthermore, the formation of ternary ion associates of lead, cadmium & mercury result in quenching of fluorescence intensity of Rhodamine 6G and the extent of quenching is directly proportional to a particular metal ion. This has been successfully employed for simultaneous multi-measurand quantification of admixtures of Pb(II), Cd(II) and Hg(II) by measuring fluorescence intensities at Stage I, Stage II and Stage III in an identical manner as described in case of colorimetric estimation. This phenomena can also be explained on the basis of the formation of J-aggregates as the emission intensities decreases proportionally at all wavelengths on addition of metal iodides, which support the formation of ion aggregates, since the ion aggregates are known to be non-fluorescent. On sequential addition of suitable sequestering agents the fluorescence intensities are enhanced proportionally.

The sensitivity threshold of fluorimetric determination is governed by two independent values, these being the analytical threshold, resulting from the intrinsic fluorescence of the reagents used in the course of the determination, and the instrumental threshold which results from instability and variable response characteristics of electronic components of the fluorimeter [Haddad, 1977]. These two thresholds may be separately estimated, and in practice the analytical threshold is the more significant of the two. Calculations show that the theoretical detection limits of fluorimetric methods are l/50-1/25 of those obtainable for equivalent photometric methods using the same ternary complexes. Practically, however, fluorimetric methods generally prove to be only about twice as sensitive as equivalent photometric methods [Babko, 1968; Haddad *et al.* 1975] although there are, of course, some exceptions. The main reason why discrepancies are observed between calculated and practical detection limits of fluorimetric methods using xanthene dye ion-association complexes is the increase in blank levels. The Schematic representation of the mechanism of fluorescence quenching showing the self- organization of receptor and fluorescent dye is shown in Scheme 6.3.



Scheme: 6.3 *Fluorescence quenching showing the competitive binding of ligands and the sequential Switching "ON"*

The use of Pyronine G instead of Rhodamine 6G has enabled individual determination of Cd(II) [Rao and Ramakrishna, 1982] and Hg(II) [Mathew *et al.* 1992].

As mentioned in the Results section, M - I^- - Pyronine G colour system also enables sequential multi-measurand detection and quantification of Pb(II), Cd(II) and Hg(II) by analogous sequential ligand exchange mechanism proposed by us in case of M - I^- - Rhodamine 6G system. Thus the present method developed is applicable using Rhodamine 6G or Pyronin G as chromoionophore which makes the system highly versatile.

6.11: ANALYSIS OF ZINC METAL, LEAD ACID BATTERY AND CHLORALKALI INDUSTRY EFFLUENTS AND NATURAL WATERS

The effluents from zinc metal, lead acid battery and chloralkali industry effluents were analyzed for cadmium, lead and mercury respectively by standard addition method using the optimized procedure described in Section 6.3.2.

Table 6.4 *Analysis of Industrial Effluents*

Industrial Effluents (Industry)	Concentration (µg mL^{-1})	
	Present method[c] (Measurand)	AAS/ FIA-AAS (Measurand)
Zinc metal[a]	75.0 ±1.4 (Cd)	88.8 ± 1.0 (Cd)
Lead acid battery[b]	< 0.06 (Pb)	0.010± 0.001 (Pb)
Chloralkali [a]	5.6 ± 0.2 (Hg)	5.7 ± 0.1 (Hg)

[a]Untreated [b]Treated [c]By standard addition method

As can be seen from the results listed in Table 6.4, the sequential multi-measurand quantification method developed in the present study provides comparable results with the standard AAS/FIA-AAS method [Ramakrishna *et al.* 1976] indicating that rapid, reliable and routine monitoring of admixture of selected heavy metal toxins is possible. The analysis of natural water such as tap, river and well water indicate that the concentrations of Pb(II) & Cd(II) were below the detection limits of 6 & 60 μg L^{-1} respectively. These results were again confirmed by standard FIA-AAS method.

6.12 CONCLUSIONS

Both multi-measurand visual detection and quantification of heavy metal toxins by colorimetry was demonstrated by advantageously utilizing sequential ligand exchange as well as fluorescence quenching. Molecular aggregation triggered by metal iodide anionic complexes resulted in J-aggregation of ion associates and subsequent red shift. Aggregates are molecular objects which occupy an important place in traditional spectroscopic research whose aim is the elucidation of spectral-luminescent changes occurring on association, the influence of this process on photophysical phenomena in solutions of dyes (the quenching of luminescence, the migration of energy), the influence on the lasing properties of solutions etc. Such template induced aggregation/deaggregation can be judiciously utilized for developing reversible optical molecular probes for continuous monitoring of various environmentally and biologically relevant species. The developed ion pair based dual optical molecular probes have good sensitivity and color stability. The applicability of the newly developed probe for rapid, reliable, routine and simultaneous monitoring of heavy metal toxins in complex environmental samples has been demonstrated. The SLE concept and dual optic molecular probes can be forerunner for establishing several other such analytical methodologies for multi-measurand quantification of admixtures inorganic/organic ions by proper choice of chromo-ionophore, pH, exchange-ligands etc using colorimetry or spectrofluorimetry.

Chapter 7

Summary of the Thesis

Environmental analysis is a potential key application for chemical sensors owing to their inherent ability to detect analytes on-line and in real time in distributed systems. There has been a pressing societal need for the development of chemo/biosensors for the detection of various analytes in solution and atmosphere, which are less expensive, simpler to construct and operate. Although biological receptors have specific molecular affinity and have been widely used in diagnostic bioassays and chemo/biosensors, they are often produced via complex protocols with high cost, requires specific handling conditions because of their poor stability. Moreover for many analytes natural receptors don't exist. Thus, there has been a strong driving force in synthesizing artificial recognition receptors. Molecular imprinting is one of the most efficient strategies that offer a synthetic route to artificial recognition systems by a template polymerization technique.

The introductory chapter envisages the relevance of monitoring of organophosphorous compounds in the environmental and the various existing techniques for its quantification. The need for the development of portable field monitoring sensors and the advantages of electrochemical methods especially potentiometric transducers over other methods have been brought out. A brief introduction to molecular imprinted polymers and a thorough literature survey on MIP based potentiometric sensors have been highlighted. Advantages of MIP based potentioselectrodes have been brought out culminating with the scope of present work to use MIP materials in sensor applications.

Chapter 2 deals with the synthesis of methyl phosphonic acid (MPA) imprinted polymer particles using methacrylic acid or 4-vinyl pyridine as functional monomers and ethylene glycol dimethacrylate as cross-linker. The design and development of imprinted polymer inclusion membrane (IPIM) based potentiometic sensor using the synthesized polymer particles has been described. The study indicates that MIPs synthesized using methacrylic acid as functional monomers show better sensing performance. The developed sensor has shown positive potential changes in the concentration range 10^{-1} -10^{-6}M with a detection limit of $1x10^{-6}$M. The developed sensor has shown higher selectivities over the analogous compounds, reversibility and reusability and has been applied for environmental samples with quantitative recoveries.

In Chapter 3 the synthesis of phorate, an organophosphorous pesticide imprinted polymer particles and the subsequent fabrication of IPIM based potentioselectrodes have been discussed. The neutral analyte has shown positive non Nernstian potential change in the concentration range 10^{-9} to 10^{-5}M with a limit of detection of 5 $x10^{-9}$M. The sensor has exhibited excellent imprinting effect over non-imprinted materials and selectivity over various coexisting analytes. From the optimization studies NPOE was selected as the better plasticizer and the ratio of MIP to PVC as 1:1 has been selected. The sensor has shown excellent detection and quantification of environmental samples.

Chapter 4 deals with the synthesis of in situ imprinted membranes based on two strategies viz. semi-covalent and non-covalent imprinting, its characterization and subsequent fabrication of the potentioselectrode. A thorough analytical evaluation of the sensor performance confirmed that the semi-covalently imprinted membrane has shown better sensitivity and selectivity. Here also the sensor has shown positive potential change in $Tris^+Cl^-$ medium for the anionic analyte. The developed sensor has been successfully used for analyzing natural water samples.

Chapter 5 gives a brief overview of the heavy metal pollution and its health effects on the ecosystem. A discussion of the conventional instrumental techniques and the advantages of low cost easily affordable colorimetric probes for its quantification have been provided. The historical development of ion associates in chemical analysis and literature survey on its application as optical molecular probes have been brought out. The scope of ion associates as optical molecular probes for multianalyte detection and quantification has been emphasized.

Chapter 6 deals with the analytical method development using cationic xanthenes dye-metal iodide ion associates as optical molecular probes for the sequential multimeasurand detection and quantification of spectroscopically silent heavy metals. The developed methodology was applicable in the range 0-100, 0-1000 and 0-200 μgL^{-1} of cadmium, lead and mercury in admixtures, with sandell sensitivities of 1.1, 15 and 2.5 μgdm^{-2} respectively. The sensing probes have shown good selectivities over other heavy metals and the proof of the method in fluorimetric sensing has also been demonstrated. The formation of aggregates of ion associates as optical molecular probes and subsequent sequential ligand exchange mechanism has been discussed. Analytical application studies on industrial samples have been demonstrated.

List of Publications

1. Molecularly imprinted polymer based potentiometric sensor for degradation product of chemical warfare agents-Part 1. Methyl phosphonic acid.
   K. P. Prathish, K. Prasad, T.Prasada Rao, M.V.S. Suryanarayana, Talanta, **2007**, 7, 11976-1980. **I.F: 3.374**
2. Design and Development of imprinted Polymer inclusion membrane-based field monitoring device for trace determination of Phorate (O,O-Diethyl S-Ethyl Thiomethyl phosphorodithioate) in natural waters.
   K.Prasad, **K.P. Prathish**, J.M.Gladis , T.Prasada Rao, Electroanalysis, **2007**,19, 1195- 1200. **I.F: 2.949**
3. Rational design of in-situ monolithic imprinted polymer membranes for the potentiometric sensing of diethyl chlorophosphate – A chemical warfare agent simulant.
   K. P. Prathish, V.Vishnu vardhan, T.Prasada Rao, Electroanalysis, **2009**, 2, 1048-1056. **I.F: 2.949**
4. Dual optoelectronic visual detection and quantification of spectroscopically silent heavy metal toxins: A multi-measurand sensing strategy based on rhodamine 6G as chromo or fluoroionophore.
   K.P. Prathish, D. James, J. Jaisy, T. Prasada Rao Anal. Chim. Acta **2009**,647, 84–89. **I.F: 3.146**

Papers Presented at National and International Conferences

1. Molecular imprinted polymer (Biomimetic) based potentiometric sensor for Phorate.
   K. Prasad, **K. P. Prathish**, J. M. Gladis, T. Prasada Rao oral presentation in the 8th International Symposium on "Advances in Electrochemical Science and Technology" held at NIO, Goa in Nov-2006.
2. Dual Optoelectronic naked-eye detection and quantification of Spectroscopically silent heavy metal toxins: A multi-measurand sensor based on Rhodamine 6G as chromoionophore.
   K.P. Prathish, D. James, J. Jaisy, T. Prasada Rao oral presentation in the National Seminar on "Analytical Chemistry- Vision 2020", ISAS, Hyderabad Chapter held at IICT, Hyderabad during March 27-28, 2008.

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