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Lead is an environmental contaminant with exposure to humans arising mostly from contaminated soil and accumulation from food. The greatest threat is to children who can retain over 30% of ingested lead whereas for adults less than 5% is retained (Martin, 1994). The effect of lead toxicity can be severe, which creates an urgent need to develop systems for its low level detection.

The development of biosensor as a method of analysis for low concentration of lead is a relatively new and promising area. Biosensors are ideal devices for analysis in the field having the desirable characteristics of simple use and cheap manufacture. They are superior to chemical sensors for metal ion detection since the recognition molecule is a biological molecule and hence could provide information on metal ion interaction with a particular organism. So the selection of biological component and transducer is the main area of investigation to develop biosensor for a particular analyte. A very extensive review on biosensors for heavy metals has been done by Verma and Singh, (2005).

2.1. Biocomponent

A promising tool for the detection of Pb (II) is the use of assay based on inhibition of various enzymes. Pb (II) can easily react with the sulphydryl (-SH) gp in a protein/enzyme which inhibits their activities and cause protein function to change.

\[
\text{Protein-SH} + \text{PB (II)} + \text{HS-Protein} \rightarrow \text{Protein-S-Pb-S-Protein} + 2\text{H}^+ 
\]

Lead is shown to have inhibitory effect on enzymes like urease, acetylcholine esterase, glucose oxidase, lactate dehydrogenase, invertase, etc. Srivastava et al. (2002) studied the kinetics of inhibition and molecular asymmetry in Pigeon pea (Cajanus cajan) urease and showed that heavy metals are potent irreversible inhibitors of the enzyme. Another example is the Aspergillus niger nitrate reductase
whose activity is drastically reduced by lead at concentrations below 10µM (Aiken et al., 2003). An enzymatic based assay for heavy metal detection was developed by Shukor et al. (2006) using papain. The assay was sensitive to Pb (II) which showed an IC$_{50}$ of 2 mM. δ-Aminolevulinic acid dehydratase (ALAD) a metalloprotein from different strains of *Pseudomonas* was also shown to be inhibited by lead. A maximum inhibition of 84.3% for *P. putida* ALAD and 81.4% for *P. pseudoalcaligenes* ALAD was observed when incubated with 750µM of lead (Korcan et al., 2007). Lead is also shown to be a potent inhibitor of peroxidase from Jerusalem artichoke (*Helianthus tuberosus L.* ) tubers with an IC$_{50}$ value of 6mM (Sat, 2008).

### 2.1.1. Urease as biocomponent

Urease (urea amidohydrolase; EC 3.5.1.5) is a hydrolytic enzyme responsible for catalytic decomposition of urea into volatile ammonia and carbamate. It is the most common enzyme used for heavy metal inhibition studies. Interestingly, both urea and urease represents landmark molecules in early scientific investigation. Urea was the first organic molecule synthesized (Andrews et al., 1984), and urease from jackbean was the first enzyme crystallized (Sumner, 1926) in addition to bring the first enzyme shown to contain nickel (Dixon et al., 1975).

### 2.1.2. Sources of urease

#### 2.1.2.1. Bacterial sources

Bacterial urease is implicated in the pathogenesis of many clinical conditions. It is directly associated with the formation of infection stones and contributes to the pathogenesis of pyelonephritis, ammonia encephalopathy, hepatic coma, urinary catheter encrustation and peptic ulceration. Several urease producing organisms have been reported to cause struvite stones in certain animals e.g. *Staphylococcus saprophyticus* is associated with stone formation in minks (Nielson, 1956).
humans, *Proteus mirabilis* is the most common organism implicated in stones formation (Rosenstein, 1986). Other urease producing species associated with infection stones include *Pseudomonas, Klebsiella and Staphylococcus sps.* (Griffith and Osborne, 1987). Other species like *Campylobacter pylori* produces ulceration (Godwin *et al.*, 1986; Mobley *et al.*, 1988). Several suggest the *Campylobacter pylori* infection is an important etiological factor in the development in the upper gastrointestinal inflammatory lesions.

In humans, approximately 20% of the urea produced by the liver is transferred by diffusion from the blood stream to the intestinal tract and hydrolyzed by urease (Visek, 1972). Now urease producing organisms are screened for enzyme production usually for biosensor application. Other bacterial sources are given in table 2.1.

### Table 2.1: Bacterial sources of urease

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Providencia rettegri</em></td>
<td>Magana – Plaza <em>et al.</em> (1971)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Griffith, (1978)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Klausner <em>et al.</em> (1980)</td>
</tr>
<tr>
<td><em>Spirulina maxima</em></td>
<td>Carvajal <em>et al.</em> (1982)</td>
</tr>
<tr>
<td><em>Proteus penneri</em></td>
<td>Krajden <em>et al.</em> (1984)</td>
</tr>
<tr>
<td><em>Brevibacterium ammoniagenes</em></td>
<td>Nakano <em>et al.</em> (1984)</td>
</tr>
<tr>
<td><em>Ureaplasma yrealyticum</em></td>
<td>Eng <em>et al.</em> (1986)</td>
</tr>
<tr>
<td><em>Bacillus pasteurii</em></td>
<td>Christians <em>et al.</em> (1986)</td>
</tr>
<tr>
<td><em>Selenomonas ruminantium</em></td>
<td>Hausinger, (1986)</td>
</tr>
</tbody>
</table>
**2.1.2.2. Fungi**

Filamentous fungi are the source of about 40% of all available enzymes such as amylases, lactase, raffinase, dextranase, pectinases and cellulose (Archer and Peberdy, 1997). Among filamentous fungi especially *Aspergillus niger* has been used for commercial production of many enzymes. Urea production and purification have been studied in *A. niger* (Smith et al., 1998). Other fungal sources in table 2.2.

<table>
<thead>
<tr>
<th>Fungi</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus tamari</em></td>
<td>Zawada and Sutcliffe, (1981)</td>
</tr>
<tr>
<td><em>Aspergillus nidulans</em></td>
<td>Creaser and Porter, (1985)</td>
</tr>
<tr>
<td><em>Schizosaccharomyces pombe</em></td>
<td>Tang and Niwa, (1997)</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>Smith et al. (1998)</td>
</tr>
<tr>
<td><em>Trichophyton rubrum</em></td>
<td>Arabatzis et al. (2005)</td>
</tr>
</tbody>
</table>
2.1.2.3. Plant sources of urease

The urease from Jack bean (*Canavalia ensiformis*) was the first enzyme to be crystallized and it remains the best characterized urease. Excessive values of urea were reported for the whole blood using concentrated soybean extract. *Canavalia obtusifolia* is nearly as such rich in urease as the Jack bean. Plant urease are mutimers of single subunit that is collinear with the three bacterial subunits. The other plant sources are specified in table 2.3.

**Table 2.3: Plant sources of urease**

<table>
<thead>
<tr>
<th>Plant</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Canavalia ensiformis</em></td>
<td>Riddles <em>et al.</em> (1991)</td>
</tr>
<tr>
<td><em>Glycine max</em></td>
<td>Polacco and Holland, (1993)</td>
</tr>
<tr>
<td><em>Citrullus vulgaris</em></td>
<td>Fahmy <em>et al.</em> (1998)</td>
</tr>
<tr>
<td><em>Cajanus cajan L.</em></td>
<td>Das <em>et al.</em> (2002)</td>
</tr>
<tr>
<td><em>Curcurbita maxima</em></td>
<td>Ibrahim <em>et al.</em> (2003)</td>
</tr>
<tr>
<td><em>Pennisetump perpureum</em></td>
<td>Bertipaglia <em>et al.</em> (2005)</td>
</tr>
<tr>
<td><em>Carya illinoinensis</em></td>
<td>Bai <em>et al.</em> (2006)</td>
</tr>
<tr>
<td><em>Vigna radiate</em></td>
<td>Naseer <em>et al.</em> (2006)</td>
</tr>
<tr>
<td><em>Cicer arietinum</em></td>
<td>Hossain <em>et al.</em> (2010)</td>
</tr>
<tr>
<td><em>Momordica charantia</em></td>
<td>Krishna <em>et al.</em> (2011)</td>
</tr>
</tbody>
</table>
2.1.3. Localization of urease

With an exception of *Helicobacter pylori*, where urease is present in outer membrane (surface localized), microbial urease appear to be cytoplasmic proteins as indicated by table 2.4.

<table>
<thead>
<tr>
<th>Micro–organism</th>
<th>Location of urease enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus pasteurii</em> (Larson and Kallio, 1954)</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td><em>Aspergillus tamari</em> (Zawada and Sutcliffe, 1981)</td>
<td>Cytoplasm (function as the storage protein)</td>
</tr>
<tr>
<td>Jack bean (<em>Canavalia ensiformis</em>) (Murray and Knox, 1977)</td>
<td>Cotyledons, in the cytoplasm of storage parenchyma cells in spherical granules and within the intracellular spaces in spherical granules</td>
</tr>
<tr>
<td><em>Bombyx mori</em> (Kurahashi et al., 2005)</td>
<td>Larval haemolymph</td>
</tr>
<tr>
<td><em>Staphylococcus</em></td>
<td>Rumen (as intracellular enzyme)</td>
</tr>
</tbody>
</table>

2.1.4. Structural characteristics of urease

Urease is structurally diverse in microbes and plant sources. Bacterial ureases are typically known to be heterotrimeric \((\alpha\beta\gamma)_3\) e.g *Brevibacterium ammoniagenes* (Nakano et al., 1984). It is also found tetrameric in *Bacillus pasteurii* (Christians and Kaltwasser, 1986) and hexameric in *Spirulina maxima* (Carvajal et al., 1982). *Helicobacter pylori* is exceptional having a dodecameric complex \(((\alpha\beta)_3)_4\) (Ha et al., 2001). The jack bean urease posses a homopolymeric \((\alpha_6)\) structure. But in all forms of urease active sites are always located in the \(\alpha\) subunit.
The information on active site of urease has been revealed by the crystal structures of *Klebsiella aerogenes* (Jabri *et al.*, 1995) and *Bacillus pasteurii* ureases (Benini *et al.*, 1998; Benini *et al.*, 1999; Benini *et al.*, 2001). The active site contains a binuclear nickel center (figure 2.1) with an interatomic distance of 3.7 and 3.5 Å in *B. pasteurii* and *K. aerogenes* respectively (Krajewska and Zaborska, 2007). In both the structures both Ni (II) ions are bridged by a carbamylated lysine through its oxygen atom. The Ni 1 and Ni 2 are coordinate to two histidines through their nitrogen atoms and Ni 2 is additionally coordinate to an aspartic acid through its oxygen atom. Both the Ni (II) ions are bridged by a hydroxide ion (WB) along with two terminal water molecules W1 on Ni 1 and W2 on Ni 2. An additional water molecule W3 is present at the opening of the active site which forms a hydrogen bonded water tetrahedral cluster with W1 and W2 in the active site cavity. This cluster is replaced by urea during the catalytic reaction. All these coordinations result in a pseudo square pyramidal and pseudo-octahedral geometry for Ni 1 and Ni 2 in which Ni 1 is penta coordinated and Ni 2 is hexa coordinated. These structural characteristics also reveals that the two ureases from *B. pasteurii* and *K. aerogenes* have superimposable active sites which is common to all ureases. In addition to the catalytic amino acid residues, those present in the mobile flap of active site also play functional role in urease catalysis. The flap functions as the entry gate for the substrate which is needed in closed conformation for the reaction. Among the amino acids in the flap Cys 319 in *K. aerogenes*, Cys 322 in *B. pasteurii* and Cys 592 in jack bean urease have been shown to participate in substrate binding, stabilizing the catalytic transition state and helps in accelerating the reaction by positioning other key residues at appropriate place in the active site. So although determined not to be essential for catalysis this cysteine molecule plays a vital role in the whole process.
and any chemical modification in the same lead to loss of mobility of the flap and the reaction is disturbed.

![Diagram of active site of urease](image)

**Figure 2.1:** a) Schematic structure of active site of urease. b) Structure of *B. pasteurii* urease (PDB 2UBP)

### 2.1.5. Urease mechanism

Urease mechanism is a debatable topic and a lot of studies had been undertaken to propose the possible mechanism. According to the studies undertaken in last decade a consensus has been reached suggesting that the initial coordination of urea to active site occur through the urea oxygen attacking the vacant coordination site on Ni (1) (Carlsson and Nordlander, 2010). But there are different views on subsequent steps. Earlier proposal (figure 2.2 A ) suggested the attack of Ni (2) terminally bound hydroxide on urea carbon which leads to the formation of a bridged intermediate of the two metal ions and release of ammonia to form product (Dixon *et al.*, (1980); Lippard, (1995). According to a second proposal (figure 2.2 B) by Benini *et al.* (2001); Musiani *et al.* (2001), the urea is attacked by the bridging hydroxide of a secondary coordination of urea nitrogen to Ni (2). This model was supported by
Pearson et al. (2000). These models suggested that the nucleophile is not the bridging hydroxide but rather a water / hydroxide coordinated to Ni (2). Recently DFT based calculations by Carlsson and Nordlander, 2010, has revealed some of the aspects regarding urease mechanism. Their study suggested that the bridging oxygen donor is a hydroxide ion which makes the structure more stable than the corresponding oxo or water complexes. The study further indicated that the binding of urea to Ni (1) through its oxygen atom directs the coordination to the trans position relative to the carbamylated lysine and lead to the release of water ligand of the resting stage. It was also observed that as per the second proposed mechanism, a urea nitrogen – Ni (2) coordination was energetically possible but further transformation including the formation of a tetrahedral intermediate based on the bridging hydroxyl group is energetically unfavorable.

![Figure 2.2: Schematic description of two proposed mechanism for urease.](image)

2.2. Enzyme immobilization

Attachment of the biological component to the transducer is vital for the success of the biosensor. It can be accomplished in a variety of ways such as covalent binding of the molecule to the detector, adsorption, entrapment, sol-gel method, etc. Das and Kayastha (1998) showed increased urease immobilization on polyethyleneimine – activated cotton cloth followed by cross – linking with dimethyl suberimidate. The immobilized enzyme had a $t_{1/2}$ of 70 days and showed practically no leaching from the immobilization matrix in 15 days. Marcos et al. (1999) reported an optical sensor in which urease was photoimmobilized with polyacrylamide onto a chemically polymerized poly-pyrrole (PPy film). The main advantage of this sensor was that no indicator dye or pH indicator was needed, because PPy itself act as the support and the indicator. Soldatkin et al. (1999) showed that the immobilization of urease under a negatively charged polymer induces an increase of the inhibition effect of the heavy metal ions due to the cation accumulation in the polymeric matrix. Based on this an enzymatic sensor was developed with field effect transistors (ISFETs) with detection limit of some heavy metal to 1mM level.

Ciurli et al. (1999) immobilized Bacillus pasteurii urease on a preformed network of Ca – polygalactouronate and showed no decrease of specific activity with respect to free enzyme. Also the immobilized enzyme showed increased temperature stability. Chen and Chiu (1999) immobilized urease covalently onto porous chitosan beads via primary amine groups connected to the backbone via a six –carbon linear alkyl spacer. Srivastava et al. (2001) immobilized pigeon pea urease on gelatin beads via cross linking with gluteraldehyde. Urease has also been electro polymerized on platinum electrode using polypyrrole and block co – polymers of thiophene – capped poly (methylmethacrylate) matrices (Alkan et al., 2001). An optimum electrolysis time of
60 mins resulted in lower $K_m$ and higher $V_{max}$ of TPMMA/PPy immobilized urease as compared to PPy immobilized urease. Jack bean urease has been immobilized onto non-porous HEMA – poly EGDMA microbeads with better kinetic properties as compared to the free enzyme. The immobilized urease retained 73% of its original activity after 75 days of repeated use. The $V_{max}$ for the free and immobilized urease was found to be $1.074 \mu M \text{mg}^{-1}$ and $3.318 \times 10^{-4} \mu M \text{mg}^{-1}$ and $K_m$ 14.49 mM and 15.94 mM respectively (Ayhan et al., 2002). Reddy et al. (2004) immobilized urease on DEAE-cellulose paper strips. In the same year urease was immobilized by covalent binding and electro polymerization to develop a conductometric urea biosensor (Hedayatollah et al., 2004). For this the platinum electrode was extensively functionalized with various reagents to get an alkyl–amino bound Pt electrode. Then chemical biding of urease was achieved using BSA and gluteraldehyde. In second method electro polymerization was done in pyrrole matrix. It was observed that the covalent binding was better method for urease immobilization as 80% of the original activity was retained over a period of 25 days and it also showed better reproducible response than electro polymerization. Mulagalapalli et al. (2007) showed the successful immobilization of urease on agar tablets, which improved the thermal stability of the enzyme to 60 °C and also increased the half life of the enzyme from 21 to 53 days at pH 7.3 and 4 °C. Urease has also been immobilized on different matrices to study their effect on immobilization efficiency and storage stability by Selvamurugan et al. (2007). They immobilized jack ban urease on nylon -6 beads, silica gel, and cellulose acetate coated gelatin film and sepharose CL 6B gel. The immobilization on poly amino matrices was mediated by ascorbic acid and it was observed that sepharose gel (94 %) and gelatin film (66 %) had maximum immobilization efficiency but the storage stability of urease was maximum in nylon.
beads followed by gelatin film, being 30 days at 4 °C. Gabrovksa et al. (2007) observed a very beneficial effect of chitosan on (Poly) acrylonitrile (PAN) membranes in terms of reduction in pore size and increase in hydrophilicity of the membrane. Urease was immobilized covalently on PAN/ chitosan membrane using gluteraldehyde and highest activity (94 %) was observed in PAN/ chitosan membrane with 0.25 % chitosan. Ahuja et al. (2008) developed a potentiometric urea biosensor based on BSA embedded surface modified polypyrrole film electrochemically deposited onto an indium-tin – oxide (ITO) coated glass plate. Whole cells of Brevibacterium ammoniagenes has also been immobilized as urease source in polystyrene sulphonate polyaniline (PSS- PAN) conducting polymer. The immobilization was done by electro polymerization on Pt electrode which resulted in 7 days storage stability at 4 °C (Kumar et al., 2009). Various immobilization matrices has been investigated for urease by Pithawala et al. (2010). They used calcium alginate beads, lac impregnated muslin cloth and paraffin wax impregnated cloth for urease immobilization. The enzyme was found most accessible to substrate in alginate and lac impregnated form with decreased $K_m$ value as compared to the free enzyme. The paraffin film was observed to mask the active sites of urease and hence showed higher $K_m$ value.

Recently Cevik et al. (2011) used Eupergit C ® macroporous beads for urease adsorption after metal chelation. The beads were functionalized with amino triazole and then used as a carrier to co- ordinate Cu$^{2+}$ ions. The Cu$^{2+}$ ions were chelated on the Eupergit C – Tri beads trough triazole group and enzyme bind the beads via co – ordinate metal. This method of immobilization showed increased $V_{max}$ and $K_m$ parameters as compared to the free system indicating an apparent low affinity of urease towards its substrate after immobilization. Yet it revealed good properties as
adsorptive beads and could be used for immobilization of other enzymes as well. Ali et al. (2011) reported electrostatic interaction of urease with ZnO nanowires which were fabricated on gold coated plastic substrate by aqueous chemical growth method. The urease was immobilized by simple electrostatic adsorption on the electrode surface in 20 mins and showed a response time of less than 4 sec with storage stability of more than 3 weeks at 4 °C. Krishna et al. (2011) immobilized purified urease from Momordica charantia seeds on chitosan beads using gluteraldehyde. The immobilization efficiency was found to be 97 % of the original and it retained 60 % activity after 14 successive enzymatic reactions. Urease has also been immobilized on poly (N – glycidyl/ pyrrole – co – pyrrole) by Bozgeyik et al. (2011) to construct an amperometric urea biosensor. A poly –phenylenediamine (PPD) film has also been electro synthesized on glassy carbon electrode by Chirizzi and Malitesta, (2011). This polymer was able to provide buffering to the enzyme and hence retained long term stability. Yurekli and Altinkaya, (2011) immobilized urease on poly acrylonitrile co – sodium methallyl sulfonate ultrafiltration membrane for different applications. The static and dynamic conditions were investigated for immobilization efficiency. It was found to enhance the thermal and storage stability of urease. Urease has also been immobilized in a thin film of silicalite and zeolite Beta on a gold electrode for conductometric measurements (Kirdekipler et al. 2011). The zeolite coated transducer avoided the use of any cross – linker for immobilization and resulted in increased response. Urease had been adsorbed along with Au nanopartical on monodispersed mesoporous silica spheres (MMSS) prepared by calcinations with complex salts (Zong et al., 2011). This method was successful in attaining pore size of MMSS in the range of 3.20 to 46.80 nm as a function of calcinations temperature. The method was simple, feasible and effective to obtain Au – urease adsorbed MMSS. Rhodium
polymer membrane has also been investigated as immobilization matrix for urease by Velichkova et al. (2011). The ammonia produced by catalytic action of urease on urea was electrochemically oxidized by rhodium hence avoided the use of second enzyme. The immobilized urease had $K_m$ of 6.5 mM and showed good reproducibility and stability. Gabrovska et al. (2011) introduced rhodium nanoparticles in PAN/ chitosan copolymer to get maximum urease activity of 77.44 %. 1 % chitosan was used to maintain the required pore size. Urease has also been immobilized on magnetic nanopartical by carbodiimide reaction (Sahoo et al., 2011). The immobilized urease was found to have better stability and catalytic activity over successive reuse.

2.2.1. Hydrosol–gel- immobilization

Sol-gel has good properties for the fabrication of biosensors. One of the main features of the sol–gel is that it can entrap biomolecules such as proteins, enzymes, antibodies, etc. The immobilized biomolecules in sol-gel matrix exhibit structural integrity, very often full biological functions and also significant stability to resist chemical and thermal deactivation. This is due to simple sol–gel processing conditions and a possibility of tailoring the process for specific requirements and providing inherent versatility. Sol–gel-derived glasses can be a potential host matrix for chemical sensing and biosensing. The sol–gel transparency with diverse chemistries and configurations for the entrapment of many bimolecular dopants also enables the development of optical biosensors. There are many advantages of using multi-layers of sol-gel films to form a stack membrane. The main advantage is to prevent a direct mixing of the chromoionophore and the urease enzyme, which may impair the activity of the enzyme. Furthermore, by using many layers of sol-gel film, the amount of enzyme for the biosensor can be increased when compared to employing a single layer of the sol-
gel film. Increasing the amount of immobilized enzyme potentially improve the response of the biosensor.

Gulcev et al. (2003) developed a urea optode containing 500 nm thick film immobilized with urease enzyme or lipase and fluorescein/carboxy – seminaphtharhodafluor-1(SNARF-1) as the sensing material. The material was conjugated to a dextran polymer backbone and mixed with hydrolyzed alkoxysilane solutions and then casted onto a planar surface to form a biologically active sol-gel derived film. Tsai et al. (2003) fabricated a urease based optical biosensor by sol-gel technique for rapid detection of heavy metals. The pH sensitive fluorescent indicator FITC dextrin was co-immobilized with urease via the sol-gel precursor tetramethyl orthosilicate (TMOS). This immobilization design allows the completion of eight measurements within 36 min automatically without affecting regeneration. In 2005, Tsai and Doong, immobilized urease in sol – gel matrix for simultaneous analysis of pH, urea, acetylcholine and heavy metals. Ilangovan et al. (2006) developed a conductometric biosensor based on sol gel immobilized urease and obtained a linear range of detection of urea and heavy metals. Another sol-gel immobilization method reported by Alqaisme et al. (2007) contained several layers (3) of sol-gel film with Nile – blue chromoionophore. This multilayer sol-gel film format enabled higher enzyme loading and a much wider linear response range of up to 100 mM urea. Pogorilyi et al. (2007) developed an efficient sol-gel method for immobilization of urease in polysiloxane layer on the silica gel surface. The activity of urease immobilized by this procedure was determined by the composition and thickness of the film, it increased upon addition of the hydrophobic (methyl) groups and polyvinyl alcohol and with decreasing thickness of the polysiloxane layer. Nepomuscene et al. (2007) used a modified sol-gel immobilization technique to develop a urease based
biosensor for determining Cr $^{2+}$ in waste water. A PVA modified silica sol – gel network was prepared for urease encapsulation by Tsai and Doong, (2007), which resulted in higher activity and stability of the enzyme. The $k_m$ of the immobilized urease was same as of free urease and the specific surface area of urease and PVA was decreased after immobilization indicating their successful co – encapsulation in sol – gel matrix. Desimone et al. (2008) immobilized jack bean urease in sol – gel derived silica nanocomposite and the effect of relative humidity on storage and enzyme activity was studied. It was observed that 80 % humidity retains maximum activity of entrapped urease when TEOS was used as silicate precursor and different additives used in the formulation effect the immobilization efficiency. Quantum Dots (QD) has also been used with sol – gel immobilization method to increase the response and shelf life of urease (Duong and Rhee, 2008). Urease immobilized in a double layer consisting of a CdSe/ZnS QD entrapped membrane and urease immobilized membrane showed minimum $K_m$ (0.1698 mM) as compared to QD entrapped membrane ($K_m$ 2.0745 mM) and urease immobilized membrane ($K_m$ 0.549 mM). The immobilized urease had storage stability of 2 months and showed that this immobilization method is suitable for other biomedical and environmental applications as well. Urease has also been immobilized in a composite sol – gel silica matrix which result in a mesoporous structure composed of closely packed spherical structures 20 -500 nm in diameter (Kato et al., 2012). The process resulted in retention of higher hydrolytic activity (90 % of the original) and storage stability.
2.3. Transducers

Biosensor construction involves various types of transducers according to the bioassay principle. In case of Pb (II), a wide number of transducers have been studied with appropriate biocomponent.

2.3.1. Electrochemical biosensors

In this system, the sensing molecule reacts specifically with compounds to be detected, sparking an electrical signal proportional to the concentration of the analyte. The most common detection method for electrochemical biosensor involves measurement of current (amperometric), voltage (potentiometric), conductance (conductometric), capacitance and impedance.

Fennouh et al. (1998) developed an amperometric biosensor with L- lactate dehydrogenase (LDH) and L - lactate oxidase co-immobilized on an oxygen electrode. The biosensor had a Pb (II) detection limit of 0.2µM and the enzyme membranes were stable for more than 2 months in 0.1M phosphate buffer at 4 ºC. A multienzyme electrochemical sensor was developed by Kukla et al. (1999) for determination of heavy metal ions based on capacitance pH - sensitive electrolyte insulator - semiconductor (EIS) sensor with silicon nitride ion- sensitive layers and different forms of cholinesterase, urease and glucose oxidase as sensitive elements. The concentration of heavy metal ions that could be detected by urease channel were within the range of $10^5$ to $10^2$ nM. Kremleva et al. (1999) also developed an electrochemical method for determining Pb (II) using an amperometric cysteine desulphhydrase tissue biosensor. The response to the action of metal was judged from a change in the catalytic activity of cysteine desulfohydrolase. Detection limit for lead was 30nM for this system. In a different transduction approach, an electrochemical enzyme biosensor for the detection of lead was developed by Veselova and
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Shekhovtsova, (2000). This sensor relied on Pb (II) inhibiting activity of alkaline phosphatase immobilized in N-phthalychitosan on polyurethane foam. The detection limit for lead was 0.10 nM. The key drawback of the sensor was the selectivity, which showed that Bi (III), Cd (II) and Zn (II) influence the determination of 0.24 nM lead. Rodriguez et al. (2004a) developed a urease-glutamic dehydrogenase amperometric assay for heavy metal screening in polluted samples. The sensor had less sensitivity towards Pb (II) which detects Pb level more than 5 X 10^7 nM. A screen printed three electrode amperometric biosensor was developed by Rodriguez et al. (2004b) based on urease and NADH-glutamic dehydrogenase system for screening heavy metals in environmental samples. They showed a 40% urease inhibition in the presence of 3 X 10^8 nM of lead after entrapment of enzyme in alginate gel on the electrode surface. It was also observed that the IC_{50} for lead in free enzyme, entrapped in alginate gel and Nafion film is greater than 50, 300 and 100 mg/l respectively. Babkina and Ulakhovich (2004) developed an amperometric ssDNA based biosensor for the study of heavy metals complexing with DNA and their determination in biological, water and food samples. The method of determination of lead is based on the preconcentration of metal ion on the biosensor followed by the destruction of DNA-metal complexes with EDTA and taking voltammogram recording. The lower limit of detection for Pb was found to be 0.1 nM. A screen printed disposable urease based biosensor was developed by Ogonezyk et al. (2005) and it was observed that a Pb (II) concentration greater than 1 mM cause <10% inhibition in 30 min. Also the non used biosensor exhibited over 95% of initial sensitivity after 4 months of storage under ambient conditions. Chow et al. (2005) developed an electrochemical sensor for the detection of lead ions by modifying a gold electrode substrate with self-assembled monolayers (SAMs) of 3-mercaptopropinoic acid (MPA) or thiotic acid (TA)
followed by covalent attachment of a lead binding peptide, human angiotensin I. A detection limit of 1nM was achieved using osteryoung square wave Voltammetry. There was no apparent loss in signals of the modified electrode after 5 regeneration and reuse cycles. An electrochemical DNA biosensor employing differential pulse Voltammetry was developed by Oliveira et al. (2008) for in situ evaluation of Pb interaction with dsDNA, the results confirmed that Pb binds to dsDNA and this interaction leads to different modifications in the dsDNA structure that corresponds to an increase of the height of the oxidation peaks of homopolynucleotides (poly A and poly G). Bagal - Kestwal et al. (2008) fabricated an inhibition based electrochemical biosensor using ultramicroelectrode (UME) for the detection of Pb. The working UME, was modified with invertase and glucose oxidase entrapped in agarose-guar gum. Lead was shown to be an irreversible competitive inhibitor with an IC₅₀ value of 112 nM and the developed biosensor had a detection limit of 3 X 10⁻⁸ M in 10 min contact time. A urease based amperometric biosensor has been developed for Hg (II) by cross-linking of urease on two different supports namely screen printed carbon electrodes and gold nanoparticles modified screen printed carbon electrodes (Dominguez – Renedo et al., 2009). The developed biosensor had a detection limit of 4.2 X 10⁻⁶ M and 5.6 X 10⁻⁸ M for Hg (II) with urease/ SPCE and urease/AuNPS/SPCE biosensor respectively. A thermostable bacterial LDH has been immobilized in a PGA pyrrole polymeric material on gold electrode (Tan et al., 2011). The biosensor showed sensitivity towards Hg and Ni at a concentration of 25 X 10⁻⁷ mM and 17 X 10⁻⁵ mM respectively. Recently an inhibition based amperometric biosensor has been developed by Do et al. (2011). The detection has been made on a urease/ NSPN/ Au/ Al₂O₃ electrode which had a sensitivity of 2980.3
µA mM$^{-1}$ cm$^2$ for NH$_4^+$ in PBS. The detection for Hg was found to be 0.05 µM with urease loading in the range of 1.06 – 2.12 U.

### 2.3.3. Conductometric biosensors

Zhylyak et al. (1995) developed a urease immobilized conductometric biosensor consisting of interdigitated gold electrodes and enzyme membranes which was able to detect lead up to 6ppm (28nM) in 10 min response time. Ilango van et al. (2006) developed a sol-gel immobilized urease conductometric biosensor on a thick film interdigitated electrode for heavy metal determination in liquid samples and showed that the inhibition of urease by lead is rapid in lower concentration range (1 X 10$^5$ – 1 X 10$^6$ nM) and as the concentration increase the increase in inhibition becomes less but shows a linear rise. The storage time of sensor was found to be 6-7 days and can be used only once to measure heavy metals. Berezhetsky et al. (2008) developed an alkaline phosphatase conductometric biosensor consisting of interdigitated gold electrodes for assessment of Pb in water. A detection limit of 193 µM was obtained and the storage stability of the biosensor in buffer solution at 4 ºC was more than one month. A three enzyme (invertase, mutarotase, glucose oxidase) based conductometric biosensor has been developed using thin film interdigitated electrodes deposited on ceramic pad as transducer (Soldatkin et al., 2012). The biosensor demonstrated best sensitivity towards Hg and Ag and could be regenerated by EDTA and cysteine.

### 2.3.4. Optical biosensors

Durrieu and Tran – Minh (2002) developed an optical algal biosensor using alkaline phosphatase for the detection of lead and showed a 70% inhibition of the enzyme in the presence of 1 X 10$^5$ nM of Pb. Kuswandi, (2003) developed an optical fiber biosensor based on immobilized enzyme for monitoring heavy metals and showed a
linear response range between $1 - 1 \times 10^4$ nM and detection limit of 2 mM for Pb. Also L-cysteine (10mM) as found to be satisfactory for regeneration of the immobilized urease.

Tsai et al. (2003) developed a sol-gel immobilized urease optical biosensor for the rapid determination of heavy metals and showed a detection limit of 100 µM for Pb. The developed biosensor showed high reproducibility and was able to complete eight measurements within 36 min. A multi analysis 50 spot array based optical biosensor was developed by Tsai and Doong (2005). The sensor was based on basic principle of inhibition of urease and acetylcholinesterase by heavy metals. Both the enzymes were co–immobilized with FITC dextran in sol–gel matrix for multianalyte detection. The biosensor had detection range from 10 nM to 100 nM for Cd (II), Hg (II) and Cu (II), but does not showed any response against Pb (II). Haron and Ray (2006) developed an optical biosensor with a three layer wave guiding silicon dioxide (SiO$_2$)/silicon nitride (Si$_3$N$_4$)/SO$_2$ structure on silicon substrate for calibration of heavy metal ions in drinking water. They observed the inhibition of urease & acetyl cholinesterase by Pb (II) and a detection limit of 4.83 nM was achieved by employing the technique of total reflection at the interface between the Si$_3$N$_4$ core and composite polyelectrolyte self–assembled (PESA) membranes containing cyclotetrachromotropylene (CTCT) as an indicator. Gani et al. (2010) had developed an optical biosensor by immobilizing urease and a pH indicator chlorophenol red in a PVC – solgel matrix for monitoring heavy metals in water samples. A liquid crystal (LC) based optical biosensor has been developed by Hu and Jang (2011). In this case urease was immobilized on a UV – tailored nematic LC called 4 – cyan – 4’ – penty biphenyl (5CB) and an orientation transition was observed from signal to dark when this crystal was immersed in urea solution. In the presence of heavy metal ion (urease
inhibitor), the optical characteristic of LC remained unchanged in urea solution. The regeneration of inhibited urease with EDTA regained the orientational transition of the LC. The biosensor is a promising tool for accurate real time monitoring of heavy metals in environmental samples.

2.4. DNAzyme based biosensors

DNAzymes also called catalytic DNA or molecular bacons have attracted the researchers because of their extreme selectivity and sensitivity towards Pb (II) ions. They have been proved to be the most efficient method of Pb (II) detection in past few years. In last decade these functional nucleic acids have become a powerful and extensively used tool for Pb (II) analysis. Relative to enzymes DNAzymes are easier to synthesize, are more stable and selective against Pb (II) ions. Motivated by the desire for simple, rapid, sensitive and portable means of quantifying low level Pb (II) contamination, recent years have seen the development of fluorescent and colorimetric DNAzyme biosensors achieving very low limits of detection. A DNAzyme based fluorosensor for Pb (II) was developed by Liu and Lu (2000) based on fluorophore (TMR) – quencher (Dabcyl) interaction present at 5’ and 3’ of substrate and enzyme strand respectively. The substrate – enzyme hybrid lead to quenching of TMR fluorescence and then cleavage of substrate strand in the presence of Pb (II) caused significant increase in fluorescence signal. However the detection limit of the developed sensor was 10nM, it showed sensitivity and selectivity at 4 °C only and an increase in temperature lead to increased background fluorescence, thus affecting the performance of the sensor. It demonstrated 80 fold selectivity for Pb (II) over other divalent metal ions at specified temperature. To overcome temperature dependent sensitivity of the fluorescent biosensor Liu and Lu (2003a) used inter and intermolecular quenchers to make the sensor applicable over a wide range of
temperature. The new design was constructed based on FRET studies of dissociated substrate strand that demonstrated a random coil conformation, much shorter than that of the full extended DNA. It also revealed the cause of increased background fluorescence in the previous sensor which was found because of dissociation of fluorophore labeled substrate strand from the enzyme strand at room temperature. The new dual quencher design suppressed background fluorescence significantly and showed an increase of 660% in fluorescence as compared to 60% for the previous sensor in the presence of Pb (II) ions. In an another work, Liu and Lu (2003b) reported gold nanoparticle assisted detection of Pb (II) ions through DNAzymes. In this case the substrate strand in the DNAzyme pair was conjugated to 13 nm gold nanoparticle (GNP) which formed blue colored aggregates after hybridization with enzyme strand. The cleavage of substrate strand by Pb (II) prevented nanoparticle aggregation resulting in red colored complex. This simple colorimetric biosensor had detection range from 100 nM to 4 µM and was successfully applied for lead detection in leaded paints with high specificity over other cations. Swearingen et al. (2005) for the first time immobilized DNAzyme on gold surface through thiolated enzyme strand. The adsorption of thiolated enzyme strand was followed by treatment with mercaptohexanol to avoid any Au – N interaction and ensure Au – S bonding for proper immobilization. This immobilization provided better sensing capability, regeneration, multiple cycle analysis and long term stability of the sensor. The detection range on gold surface was from 1 nM to 10 µM and lowest detection limit was 1 nM. At the same time a microfluidic – nanofluidic hybrid device was constructed by Chang et al. (2005). In this case substrate strand was labeled by a fluorophore at the 5’ and the the enzyme strand at 3’ end by a quencher. The detection was done in a spatially confined space in the nanofluidic device using laser induced
fluorescence. The biosensor had a linear range of 0.1 – 100 µM and a detection limit of 11 nM. The developed biosensor was applied for lead detection in electroplating sludge reference material and demonstrated high selectivity for Pb (II) ions. Wernette et al. (2006) immobilized DNAzyme in a Au – coated polycarbonate track – etched (PCTE) nanocapillary array membrane (NCAM) through thiolated enzyme. The study was based on a ratiometric method where a non – cleavable substrate strand (devoid of RNA base) was used for ratiometric comparison of intensities. This method provided real time monitoring as well as allowed substrate based standardization for more efficient detection. The thiol gold immobilization of enzyme strand was followed by fluorophore labeled substrate binding and then cleavage by Pb (II) ions. The cleaved fluorophore in the solution was then detected for lead quantification. The constructed sensing structure could be stored for 30 days and provided a detection limit of 17 nM. Xiao et al. (2006) concentrated their work on electrochemical detection of lead using DNAzymes to overcome various limitations of optical measurement. According to them the optical method suffers from drawbacks like false signal due to colored contaminants or fluorescence interfering molecules and require costly optical devices. Electrochemical methods on the other hand have the advantage of miniaturized assembly and relatively scarcity of electroactive contaminants. The detection was based on the conjugation of methylene blue on the catalytic enzyme strand that after the cleavage of substrate strand was free to transfer electrons to the gold electrode on which the whole DNAzyme complex was chemi – absorbed via thiolation. The detection range of the sensor was from 0.5 to 10 µM with a detection limit of 0.3 µM after an incubation of 1 hr at 37 °C. DNAzyme has also been entrapped in sol – gel derived matrices and applied for metal ion sensing (Shen et al., 2007). The entrapment showed that all DNAzymes retained their
partial catalytic function in silica based hydrophobic or hydrophilic materials but with increased response time and low signal enhancement. Maximum sensitivity towards metal ions was observed in amalgamate containing 40 % MTMS and 60 % TMOS. The study demonstrated that both TMOS and MTMS lead to low signal enhancement and momentous leaching respectively. Thus a hybrid silica material was prepared for entrapment that reduced signal interference and improved biosensing performance.

Wei et al. (2008) reported a simple, sensitive and label- free 17E DNAzyme based sensor for Pb (II) detection using unmodified gold nanoparticles (GNPs). They modified the Au nanoparticle based biosensor developed by Liu and Lu (2003b) to make it simpler and cost effective. The specific property of citrate protected GNPs to adsorb ssDNA instead of dsDNA was utilized to construct Pb (II) sensitive biosensor. The unfolded ssDNA produced after the cleavage of substrate strand by lead action were able to stabilize GNP in the presence of high concentration of NaCl whereas dsDNA could not. This lead to GNP aggregation in the latter case and hence change in colour from red to blue. A detection limit of 500 nM in 20 mins was realized by this method with high selectivity and sensitivity over other metal ions.

Now a new era of DNAzyme based sensors has arisen in which new molecular probes (G rich oligonucleotides and G quadruplex) has been optimized with some modifications to give better signals with Pb²⁺ ions. Taking up the same method, Elbaz et al. (2008) developed a DNAzyme cascade based biosensor using HRP mimicking DNAzyme along with the Pb (II) dependent DNAzyme. The detection was based on a cascade reaction that was initiated by cleavage of substrate strand by Pb (II). The product of this reaction lead to formation of a catalytic G - quadruplex that catalyzed the oxidation of luminol by H₂O₂ and produced chemiluminiscence. The whole process was possible only by tethering two nucleic acid sequences, partially
complementary to HRP mimicking DNAzyme at the 5’ and 3’ end of the substrate strand. The cleavage of substrate strand by Pb (II) released HRP mimicking DNAzyme sequences that intercalated in a G quadruplex structure in the presence of hemin and carried out oxidation process resulting in a colored product. Hence the Pb (II) analysis could be done through colorimetric assay of the released HRP mimicking DNAzymes. The detection limit of the sensor was 10 nM and had a magnificent selectivity and specificity for Pb (II) ions in the presence of other cations. Zhang et al. (2010) has utilized the potential of DNAzymes as catalytic and molecular beacon to achieve a detection limit of 0.6 nM. The substrate strand has been modified to form an intermolecular beacon which has a fluorophore at one end and a quencher at the other. The cleavage of the substrate strand leads to the separation of the quencher and the fluorophore and hence increase in the fluorescence. Li et al. (2010) has developed a Pb$^{2+}$ ion detection system based on peroxidase like activity of K$^+$ stabilized G quadruplex DNAzyme named PS2.M with two detection means. The K$^+$ stabilized PS2.M binds to hemin with high efficiency forming a complex that mimics horseradish peroxidase (HRP) and catalyzes H$_2$O$_2$ mediated oxidation of 2,2’ – azino – bis (3 – ethylbenzothiazoline-6 – sulfonic acid) diammonium salt (ABTS) and luminal to generate color change or chemiluminescence (CL) emission. But the addition Pb$^{2+}$ ion leads to some conformational change in the PS2.M quadruplex which then does not bind to hemin. This conformational transition is accompanied by decrease in DNAzyme activity and therefore less change in color and CL emission. Pb$^{2+}$ ion could be detected upto 32 nM colorimetrically and 1 nM using the CL emission method. In continuation to the exploitation of G - quadruplex complexes Li et al. (2010) reported a very efficient and reusable duplex – quadruplex exchange based nanodevice that included the selectivity and sensitivity of a Pb (II) driven G
quadraplex assembly. This DNA device consisted of duplex formed by a hybrid of oligonucleotides sequence called T30695 and complementary strand called X. the addition of Pb (II) ions induce unwinding of the duplex and lead to folding of T30695 into a G – quadruplex (G4) structure stabilized by Pb (II) ions. The formation of G4 structure is realized by the increase in fluorescence of zinc protopor - phyrin IX (ZnPPIX) after binding to G4. The ZnPPIX enables fluorescence detection without the need of any fluorophore labeling. The whole structure could be detangled by DOTA for repeated use. DOTA chelates Pb (II) from the complex and reforms the duplex assembly. This specialized DNA nanodevice had a linear range from 20 nM to 1 µM with lowest detection of 20 nM. In an ease to develop a more sensitive sensor Li et al. (2011) has now developed a sensor in which Pb^{2+} ions induces formation of G4 quadruplex of AGRO100 molecule that can bind to hemin to form complexes. This G4 AGRO100 – hemin complex can effectively catalyze the H_{2}O_{2} mediated oxidation of amplex UltraRed (AUR), resulting in increase in fluorescence. The detection limit achieved by this device was 0.4 nM. Lately Wang and Irudayaraj, (2011) developed a SERS based biosensor using DNAzyme assembly. In this study the substrate strand was conjugated with gold nanoparticles of 45 nm which were further attached to raman reporters to produce raman signals. The enzyme strand was immobilized on gold surface through thiolation followed by hybridization with GNP labeled substrate strand. The cleavage of substrate strand by the addition of Pb (II) lead to cleavage of GNP conjugates from the gold surface resulting in decreased raman signal. The decreased number of GNPs at the gold surface was related to the SERS intensity of raman reporters and could be used as effective indicators of Pb (II) ion concentration. The SERS DNAzyme biosensor has high selectivity and sensitivity toward Pb (II) ions with a detection range of 20 nM – 1 µM and lowest detection limit
was 20 nM. Recently, Ma et al. (2011) developed an electrogenerated chemiluminescence (ECL) biosensor based on the use of ruthenium complex tagged 5’- amino -17 E’ as an ECL probe. The modified Ru 1-17 E’ and substrate strand were covalently coupled on a graphite electrode modified with 4 –aminobenzoic acid. Pb2+ ions induced cleavage of substrate strand leads to dissociation of double stranded DNA complex and hence high ECL signal. The detection limit achieved by the workers is 1.4 pM. Till now DNAzyme has been applied for monitoring lead in drinking water and leaded paints. The authors work is the first endeavor in which DNAzyme has been applied for monitoring lead in milk.

2.5. Recombinant microbial biosensors

Development of recombinant microbial sensor has been an intriguing subject of research now days. rDNA technology has lead to the development of microbes which react to the presence of lead by expressing reporter genes inserted. Using the same technique Tauriainen et al. (1998) developed a luminescent bacterial sensor for lead by inserting the regulation unit from the cad A determinant of plasmid p1258 to control the expression of firefly luciferase. The expression of reporter genes as a function of added extra cellular heavy metals was studied in Staphylococcus aureus strain RN 4220 which responded to lead with lowest detectable concentration of 33nM. These results were obtained with only 2-3 hrs incubation time. In a continuation of this work, Tauriainen et al. (2000) used freeze dried luminescent bacteria as chemical reagents to detect the bioavailability of toxic metals from spiked natural water samples and showed a slight decrease in signals due to insolubility of lead in the water samples. The decrease in signals was also due to some turbidity causing compounds and increased salinity of the water samples. Ivask et al. (2004) reported the use of recombinant luminescent bacterial sensor for the measurement of
bioavailability of lead in soils polluted by metal smelters. Liao et al. (2006) developed a green fluorescent protein (GFP) based bacterial sensor *E. coli* DH5α (pVLCD 1). DH5α (pVLCD 1) responded to Cd (II), Pb (II), and Sb(III), the lowest detectable concentration being 0.1nmol/l, 10nmol/l and 0.1nm/l respectively with 2h exposure. Lately Chakraborty et al. (2008) had reported a bacterial biosensor that responds to Pb (II) in the range of $5 \times 10^4 - 4 \times 10^5$ nM by expressing GFP. The genetic element that senses lead includes the regulatory protein gene (PbrR) along with operator/ promoter (PbrO/P) of lead resistance operon from plasmid pMOL30. PbrO/P also controls the gfp reporter gene expression. The host used was *E. coli* DH5α. It was observed that the GFP respond to induction by lead peaked at $2.5 \times 10^5$ nM and then declines. The biosensor was tested for co-inducibility for Cd (II), Zn (II) and Hg (II). Only Zn (II) showed mild induction at high concentrations and the highest fluorescence obtained was 8.5 times lower than that obtained with Pb(II). This makes the biosensor specific for lead.

**2.6. Miscellaneous biosensors**

Blake et al. (2001) developed an antibody biosensor which could detect lead-complex upto 4.83 nM in 10 min response time. Liu et al. (2007) developed cardiac cell based biosensor for the detection of heavy metal toxicity showing a characteristic change in beating frequency and amplitude of cardiomyocytes in the presence of 10 µM Pb (II), in less than 15 min. Yin et al. (2007) developed a piezoelectric biosensor for the detection of lead ions in solution by immobilizing BSA on to a colloidal Au- modified piezoelectric quartz crystal. The detection limit of 1 nM for Pb (II) was obtained and the sensor chip could be regenerated by dipping in EDTA solution for approximately 2hrs. The chip could be used to detect Pb (II) for eight times without obvious signal attenuation.
Another class of sensors for heavy metal detection comes under electrochemical sensors which are devoid of any biological component and are worn as analytical tools for heavy metal detection. These mainly comprises the stripping voltametric studies on different kind of modified working electrodes which have now publicized as a fast method of heavy metal detection with low limits of detection. They also have the advantage of differentiating different metals based on their polarization potential. For example Pb(II) and Cd(II) determination and their interaction studies has been done on boron doped diamond electrode by differential stripping voltametry (Manivannam et al., 2004). This technique enabled simultaneous analysis of Pb(II) and Cd(II) in the range of 1 – 5 µM with separate stripping peaks. Similarly simultaneous study of Pb (II) and Cd(II) has also been done in milk through differential pulse polarography by Tokusoglu et al. (2004). Standard addition method has been used to detect Pb (II) in a range of 0.92 – 1.42 µM. Liu et al. (2005) used carbon nanotube nanoelectrode arrays coated with bismuth film for voltametric detection of Pb(II) and achieved a detection limit of 0.2 nM. A detection limit of 9.66 nM has been achieved by Hassan et al. (2008) with carbon paste electrode modified with chitosan. A zeolite NH$_4$ – Y modified carbon paste electrode was able to achieve the detection limit 17.4 nM (Senthilkumar and Saraswati, 2009). Recently Bouwe et al. (2011) developed an electrochemical sensor using 1,10- phenenthroline montmorillonite intercalate on carbon paste electrode and achieved a detection limit of 0.4 nM by adsorptive stripping voltametry.
2.7. Biosensors developed for Milk adulteration

2.7.1 Urea biosensor

Urea is one of the major contaminant in milk now days owing to the manufacturing of household synthetic milk. A number of dairies are manufacturing synthetic milk by mixing urea and detergents. Urea is not a natural constituent of milk and is considered as an adulterant. Its presence in milk of dairy cattle could be attributed to excessive nitrogen uptake and the rumen efflux of crude protein intake. The above factors can account for the presence of urea in the range of 1.66 – 2.66mM (low milk urea nitrogen MUN) and 2.83 – 4.16mM (high MUN). The synthetic milk can contain a very high concentration of urea which is very harmful to kidney functions and effects normal functioning of many organs. So there is a great need to develop a simple and cost effective analytical tool for detecting urea and hence adulteration in milk. A very prominent tool is the development of urease based urea biosensor. A lot of biosensors have been developed for sensitive estimation of urea but, a few has applicability on milk samples. Jenkins and Delwiche, (2002) developed a biosensor based on a manometric assay of the carbon dioxide generated by the enzymatic hydrolysis of urea for on-line measurement of urea in milk during milking. In this assay, urea was hydrolyzed by urease to ammonium and carbonate and then enclosed in a sealed cell with citric acid to allow the carbon dioxide to be liberated from solution. This creates a change in pressure in the cell, which could be measured and related to urea concentration in the sample. Verma and Singh, (2003) developed a potentiometric microbial biosensor for urea detection in milk using immobilized urease yielding *Bacillus sp.* as the biocomponent. The developed sensor showed a good correlation of results regarding urea detection with pure enzyme system and spectrophotometric methods with response time as low as 2 min. Renny *et al.*, (2005) developed a urease
based sensor for the detection of urea in milk using a piezoelectric sensor, which measures the pressure of the gas evolved in the sample. The sensor showed linear behaviour for varying concentration of urea in the sample and the response time was 3 min. A potentiometric biosensor for urea has been developed by immobilizing urease on to the ammonium ion selective electrode using a polymer composite of poly (carbamoyl sulphonate) and polyethyleneimine (PE). The biosensor has a detection limit of $2.5 \times 10^{-5}$ M and has been successfully applied on milk samples (Trivedi et al., 2009). Mishra et al. (2010) has developed a flow injection based urea biosensor by covalently immobilizing urease on controlled pore glass (CPG) and then packing in to a column inside thermistor. The urea hydrolysis was monitored in terms of specific heat produced in the system. For application on milk samples a simple filtration and matrix matching technique was done prior to analysis. The biosensors had a linear range of 1- 200 mM under standard conditions and operational stability of 3 months.

2.7.2 Aflatoxin biosensor

Aflatoxins comprise a group of mycotoxins produced by some strains of *Aspergillus flavus* and *A. Parasiticus*. They are defamed because of their hepatotoxic and carcinogenic effects and are found in many food and dairy products including milk. Aflatoxin contamination in milk could take place by two means; firstly either by ingestion of aflatoxin contaminated feed by the animal and its subsequent transfer in the milk or through contamination of milk with fungi. In both cases it makes the milk unhealthy for intake and need to be detected at right stage. A number of aflatoxins mainly B1, B2, G1, G2, M1 and M2 have been grouped as effective hazardous compound. The European commission and Codex Alimentarius commission has set a MRL of 50 ng l$^{-1}$ AFM 1 in milk. In a study conducted on Indian infant milk products
and liquid milk samples by Rastogi et al. (2003), 75% of milk samples exceeded the MRL for AFM1 in milk. The extend of AFM 1 contamination was found to be higher in infant milk products (65-1012 ng l⁻¹) then the liquid milk (28-164 ng l⁻¹). A maximum contamination of 127.6 ng kg⁻¹ was found in the milk samples of Turkey, indication 64% of the samples exceeding the regulatory limits (Celik et al., 2005). A study in southern Italy on AFM1 contamination in cheese reported 31.3 % and 27.2% contaminated cheese samples made from mixed sheep-goat and cow milk respectively depicting the subsequent milk contamination by the aflatoxins (Montagna et al., 2008). In Iran a contamination level in the range of 7- 476 ng l⁻¹ was found in tested liquid milk samples and 10% infant milk formula (Rahimi, 2010).

To avoid the cases of aflatoxicosis various biosensors has been developed for its sensitive detection. Talking from last decade the first successful biosensor for aflatoxins detection was a flexible, small, reliable, highly sensitive and fully automatic hand held immunoaffinity fluorometric biosensor. It was a combination of electronics and fluidics and had a detection range from 0.1 to 50 ng ml⁻¹ with a response time of less than 2 minutes (Carlson et al., 2000). An electrochemical immunosensor was developed against AFM 1 by immobilizing the antibodies on screen printed electrodes (SPE). Chronoamperometry was performed at 100 mV for sensitive analysis of AFM 1. A detection range between 30- 160 pg ml⁻¹ and a detection limit of 25 pg ml⁻¹ was achieved in this case (Micheli et al., 2005). In continuation to development of electrochemical biosensors, Parker et al. (2009) constructed an electrochemical immunosensor against AFM 1 by immobilizing antibodies on SPCE. Competitive ELISA assay was performed on the surface of working electrode using TMB/ H₂O₂ reaction. The most important investigation in the study was the removal of interference due to α - lactalbumin by incorporating 18 mM
CaCl$_2$ in 1:1 ratio to milk. The developed immunosensor had a detection limit of 39 ng l$^{-1}$ and proved to be better than HPLC and ELISA Kit. In an effort to develop low range electrochemical immunosensor Paniel et al. (2010) developed a competitive immunoassay based biosensor. The recognition molecules along with the tracer were deposited over a SPCE to measure enzymatic response amperometrically. The developed biosensor had a detection limit of 10 pg ml$^{-1}$. A novel biosensor based on fusion protein has been constructed for AFB1. The fusion protein was prepared by genetically fusing gold binding poly peptide to protein A (Pro A), which was used as cross linker for antibody immobilization on SPR substrate. The GPP –ProA Protein was proved to be effective cross linker and enabled a detection limit of 10 ng l$^{-1}$ AFB 1 ( Ko et al., 2010 ). A DNA biosensor was constructed for the impedimetric biosensing of AFM 1 using thiol modified single stranded DNA probe capable of binding to AFM 1 (Dinckaya et al., 2011). Electrochemical impedance spectroscopy and cyclic voltametry were used to monitor layer by layer deposition of cystamine, gold nanoparticle and ss- HSDNA on gold electrodes. The biosensor achieved a linear response in the range of 1-14 ng l$^{-1}$ and was applicable to real milk samples.

**2.7.3 Antibiotic biosensor**

Antibiotics given to the lactating animals as veterinary drugs lead to their entry in milk and pose a high risk of resistance development in humans over continuous intake. So their regular check in milk samples is extremely important to confer milk purity. An optical biosensor was developed by Ferguson et al. (2002) based on inhibition immunoassay for streptomycin and dihydrostreptomycin residues in milk (MRL 200 ng ml$^{-1}$). A reusable stable sensor chip was developed for multiple analyses and the detection limit achieved was 30 µg kg$^{-1}$. A SPR based inhibition assay has been developed by Sternesjo and Gustavsson, (2006) for detection of β -
lactams in milk. Carboxypeptidase has been used to hydrolyze a tri peptide in to a di peptide, a reaction inhibited by β - lactams. The amount of substrate utilised or the product formed was monitored by using polyclonal antibodies against substrate and product respectively. The inhibition assay had a detection limit of 1.2 µg kg⁻¹ for penicillin G (MRL 4 µg kg⁻¹) in milk and was found to be comparable to commercial kits in terms of performance and accuracy. Zacco et al. (2007) has developed an electrochemical immunosensor for antibiotic detection in milk by immobilizing anti-sulphonamide antibodies on to tosyl- activated magnetic beads. Simple covalent binding was found to be best for immobilization. The use of magnetic beads enabled easy electrochemical immunosensing through magneto sensor used to capture magnetic beads and subsequent antibiotic analysis. The sensor has a detection limit of 1.44 µg l⁻¹ and was rapid, cost effective than other methods. Another sensitive wavelength interrogated optical sensor has been fabricated as a miniaturized device for antibiotic detection in milk. The microfluidic has been combined with competitive immunoassay to develop a lab on a chip based method for multi antibiotic analysis. The three antibiotic families were tested in a response time of 10 minutes with 95% accuracy for their respective MRLs in milk (Suarez et al., 2009). To achieve multi analyte profiling in milk along with sensitivity a SPR based immunosensor has been developed by Rebe - Raz et al. (2009). The single sensor chip was able to detect different classes of antibiotics simultaneously. This micro array biosensor was used to perform seven immunoassays in a competitive manner to achieve ng l⁻¹ level detection limit. Another SPR based biosensor has been developed by Keegan et al. (2009) to detect a veterinary drug Benzimidazole Carbamate (BZT) residue in milk. A solid phase extraction clean up method was used to extract BZT residues from milk and then applied to SPR using polyclonal antibodies developed against BZT – protein
The detection limit achieved was 2.7 µg kg⁻¹ which was lower than the MRL set by EU (10 µg kg⁻¹). A recombinant whole cell biosensor was developed for tetracycline testing in milk by Scaria et al. (2009). The recombinant called *E-coli* JM 109 (pJSKV 41) had a fusion of tet R regulated tet promoter from a plasmid pOT182 and enhanced green florescent protein (EGFP) gene. The whole cells of recombinant *E. coli* were able to detect tetracycline in the range of 10 – 60 ng ml⁻¹ and oxytetracycline 25-125 ng ml⁻¹ against their MRL 100 ng ml⁻¹. In continuation to SPR technology another SPR based biosensor was developed for multianalyte antibiotic detection in milk by Fernandez et al. (2010). A portable six channel SPR device was used for label free and simultaneous detection of enrofloxacin, sulfa pyridine and chloramphenicol residues in milk and the detection limit achieved were 0.3 µg l⁻¹, 0.29 µg l⁻¹ and 0.26 µg l⁻¹ respectively. Chen et al. (2010) has developed a very effective amperometric biosensor for penicillin. In this case β - lactamase, hematin (pH sensitive redox probe) and multiwall CNT were co immobilized on glassy carbon electrode layer by layer for sensitive amperometric detection of Penicillin V upto 19 µg l⁻¹. This biosensor was found to be better than the commercially available pH change based biosensor. A novel aptamer based electrochemical biosensor has been developed by Zhang et al. (2010) for rapid determination of tetracycline in milk. The aptamer used was highly specific for tetracycline and was immobilized on surface of GCE for electrochemical analysis of upto 1 ng ml⁻¹ in a response time of 5 minutes. Recently an inexpensive and portable bio sensor has been developed for floroquinolone (FQ) detection in milk (Fernandez et al., 2011). The SPReeta three channel gold chip has been used to immobilize FQ haptenized protein. This method involved simple fat removal and dilution step for milk samples to attain a detection limit of 2 µg l⁻¹.
2.7.4 Pesticide biosensor

Pesticides due to their extensive use in modern agriculture practices pose a high risk of contamination in human food chain. Their bioaccumulation and fast biodegradation in more toxic compounds represents a high risk to human health and environment. They have also been found as contaminant in milk due to leaching from soil to water bodies surrounding the lactating animals. So a fast and simple detection tool is needed for their sensitive analysis. Zhang *et al.* (2005) has developed a disposable biosensor to detect organophosphate and carbamate pesticides in milk. A recombinant acetyl cholinesterase with enhanced sensitivity was used for inhibition assay on screen printed thick film electrodes. The biosensor was able to detect carbaryl up to 20 µg l⁻¹ and paraoxon up to 1 µg l⁻¹ in milk samples without any pre treatment. Mishra *et al.* (2010) has reported a high – through put enzyme assay for organophosphate detection in milk. The analysis was based on butyrylcholinesterase inhibition by the pesticide. The method has been applied to 384 well plates and then miniaturized to 1536 well plate. The detection limit obtained for OP in milk was 1 ng l⁻¹ in 384 well plates in 12 minute time period which was lowered down to 0.5 ng l⁻¹ in 1536 well plate within 5 minutes response time. Recently an amperometric biosensor has been reported by Chauhan and Pundir (2011) for OP measurement in water and milk samples. The detection was based on inhibition of acetylcholine esterase which was immobilized on a gold electrode covalently through iron oxide nanoparticle and carboxylated MWCNT. The detection limit for malathione and chlorpyifos was 0.1nM and for endosulfan 10 nM.

2.7.5. Biosensors for other contaminants in milk

Haasnoot and Du Preg (2007) had developed a cost effective immunosensor for detection of non milk proteins in milk powder. An optical biosensor based on
immunoassay for major non milk proteins i.e soy, pea, and soluble wheat proteins has been constructed. Simultaneous detection in different flow channels of the biosensor chip through polyclonal antibodies was done to achieve a detection limit of 0.1 % plant protein in total milk protein content.

Casein which is thought to be a potent allergen present in milk has also been explored for biosensor development (Hiep et al., 2007). An LSPR based immunosensor has been developed to check its content in milk. A three layered substrate comprised of top and bottom gold nano particle layers carrying silica nano particles layer in between was used for the detection. The detection limit achieved was 10 ng ml\(^{-1}\) and the biosensor proved to be cost effective compared to other available biosensors.

An amperometric bio sensor has been developed for lactose determination in milk (Conzuelo et al., 2010). The sugar hydrolyzing enzyme beta galctosidase, glucose oxidase, peroxidase and a mediator called tertrathiafulvalene (TTF) were co immobilized on MPA – SAM modified gold electrode for the detection. A cascade of enzymatic reactions produced an amperometric signal through redox mediator TTF and showed a linear range over 1.5 X 10\(^{-6}\) to 1.2 X 10\(^{-4}\) M with detection limit 4.6 X 10\(^{-7}\) M.

Presence of unwanted bacterial consortia in milk is the most common type of contamination found which indicate unhygienic milking practices and afterward handling of milk. An impedance based portable biosensor has been developed by Lanzoni et al. (2011) to minimize the time period required for microbial screening through standard plate count technique which usually takes 24 to 72 hours. This biosensor had a response time of 3 – 12 hours in raw milk and also has the advantage of on field application.
2.8. National status

In India till now no one has reported the development of biosensor for specifically monitoring lead in milk. The Biosensor Technology Lab, Department of Biotechnology, Punjabi University, Patiala is the foremost group to work on this project under the supervision of Prof. Neelam Verma.