Development of Novel Biosensing Micro Techniques for Analysis of Pesticide Residues and Heavy Metals in Water and Milk

SYNOPSIS

Submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

RUPESH KUMAR MISHRA

Under the Supervision of

Prof. Sunil Bhand

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

PILANI (RAJASTHAN) INDIA

2012

1. Introduction

Excessive contamination of various environmental components leads to pollution of the biosphere and eventually reaches humans via the food chain. In particular, contamination of milk by toxic substances such as organophosphate residues (OPs) and trace heavy metals (HMs) causes a serious problem to the agricultural and dairy industry. Various agencies have set stringent guidelines such as the European Union (EU) has set maximum admissible concentrations of 0.1 μg L^{-1} for individual and 0.5 μg L^{-1} for total pesticide residues (EU Commission Directive1999/50/EC) and 1 μg L^{-1} for mercury (Hg^{2+}). Biosensors and biochip have emerged as a promising technique for screening of OPs residues and HMs to meet the stringent regulatory standards. This thesis encompasses development of novel biosensing micro techniques for analysis of OPs residues and HMs based on native, stabilized and genetically modified enzymes as biocomponent and chemiluminescence (CL) and amperometry as a detection technique.

2. Knowledge gaps in the existing literature

2.1 Need for ultrasensitive, highthroughput and high sensitivity bioassay for OPs analysis: OPs are neurotoxic even when present at ultra low concentrations. Conventional analytical techniques do not meet stringent regulatory standards for monitoring their levels in water and milk; they are very slow and provide low sample throughput. Highthroughput techniques exploring enzyme assay using 384 and 1536 well plate format has been reported in the area of clinical sciences, whereas it is yet to be explored in the area of environmental analysis. Stability of biomolecules is also one of the major limitations for biosensor development.

2.2 Need for sensitive miniaturized biochip based detection systems for OPs and HM: Micro and nanofabrication technology has facilitated development of biochip based devices. Miniaturized biochips are getting attention owing to their, higher sensitivity, portability, small sample volumes and low volumes of hazardous waste. Using biochip, a number of samples can be simultaneously analyzed, or multiple assays can be run on a single platform. The powers of on-chip enzymatic assays offer enhanced sensitivity with great promise for point-of-care testing

for real samples such as water and milk. Thus there is a need for high sensitivity screening assay to determine such toxic contaminants at low level.

2.3 Development of biosensor for multianalyte determination: In environmental compartments, samples may contain multiple pesticides or HMs (Laetz et al., 2009). Hence, it is essential to evaluate the synergistic effect of toxic contaminants co-existing with analyte of interest. Thus, there is a need for novel biosensing techniques which can provide information on toxic effect of mixture of OPs residues or multiple analytes.

2.4 Development of online determination and detoxification of OPs in milk: Recent survey of the literature has also revealed that there is an urgent need for automated on-line monitoring of OPs in milk. The dairy industry is one among them, where continuous evaluation of milk constituents and milk contaminants is of utmost importance. Degradation of OPs is very mandatory for the detoxification of waters and milk devoted to human consumption (Istamboulie et al., 2010). Significant advances have been made in the development of analytical protocols using novel strategies. However, there are few reports on application of enzymatic assays for monitoring the removal/detoxification of toxic contaminants.

3. Scope of the work

The increasing exposure of potentially harmful pollutants such as metals and pesticide residues to human population calls for sensitive, fast and cost-effective bio-analytical techniques. Novel high-throughput micro techniques capable of detecting these analytes at the limit set by regulatory agencies in various matrices such as milk can be achieved using biosensor/biochip. Development of biochip based system facilitates field deployable techniques capable of analyzing trace analytes in contamination site. For online detection and detoxification of OPs, flow based biosensors are needed. Application of enzyme biosensor for monitoring removal of contaminants is few in the literature. The following objectives were set to fulfill the gaps:

4. Objectives of the work

1. Development of novel high-throughput enzymatic optical biosensor for analysis of pesticide

residues such as OPs and carbamates (CM) in matrices such as water and milk.

2. Development of novel miniaturized enzyme based biosensors/biochip for pesticide residues and HMs.

3. Develop innovative biosensors for monitoring removal of contaminants such as OPs and HMs.

5. Methodology adapted

A highly sensitive and highthroughput enzyme assay was developed for OPs determination in milk using enzyme inhibition based principle. A novel butyrylcholinesterase (BuChE) stabilization protocol was developed. BuChE and methyl paraoxon (MPOx) were used as a biocomponent and model inhibitor and CL technique was employed. The recoveries were checked in milk to evaluate the developed assay (chapter 2). A novel protocol was developed using surface modification for immobilization of acetylcholinesterase (AChE) on chip. The biochip was tested for determination of MPOx, ethyl paraoxon (EPOx), methyl parathion (MP) and carbofuran (CF) in milk using CL technique. The AChE showed an excellent stability when coupled on chip and stored at room temperature for more than 3 months (chapter 3). Glucose oxidase (GOx) was immobilized on novel nanostructures. Novel aluminium nano structures have been demonstrated as simple and cost-effective platform for Hg^{2+} biosensing. The device facilitates high-throughput analysis of Hg^{2+} in 20 minutes using CL technique (chapter 4). A visual colorimetric assay was developed for both OPs and CM using alkaline phosphatase (ALP). The combination of ALP and functionalized SiO_2 as a biosensing platform for OPs determination was used. The technique is free from interferences of HMs at low level, affordable to common population and simple to perform and interpret the results visually (chapter 5). A novel automated flow based biosensor was developed for detection and detoxification of OPs. The genetically engineered AChE enzymes variants; B394, B4 and B131 were immobilized on screen printed electrode and integrated on flow cell to detect OPs residues.

6. Conclusions

Bioassay and biosensors for OPs and HMs have been successfully developed in water and milk using optical and amperometric techniques. The highthroughput and miniaturized assay for OPs

and HM using micro well plate and biochip device has been successfully demonstrated in this thesis. A novel automated flow based biosensor was developed for detection of OPs using B394 and B131 and B4 and detoxification of OPs using phosphotriesterase (PTE). Chapter wise conclusions are as under;

Chapter 1 Introduction Detailed description of biosensors, classification of biosensors, enzyme based biosensor, various aspects of biosensors were presented. State of the art for inhibition based biosensors for analysis of OPs and emerging trends in biosensors were also discussed. Gaps in the existing research and objectives of the work were also described.

Chapter 2 Highthroughput enzyme assay for analysis of OPs residue in milk The significance of the demonstrated work lays in the facilitation of microplate based high-throughput, sensitive and economical bio-assay for determination of OPs residues. The BuChE based bioassay enabled low level determination of individual OPs in milk sample with limit of detection (LOD) 0.001 µg L^{-1} using CL technique. The assay is based on the inhibition of enzyme BuChE for quantification of OPs residue in milk. BuChE was stabilized and preloaded in 384 well plates at 30 ºC. The assay permits rapid determination of OPs in milk within 12 min. The enzyme assay was tested for individual and mixture of OPs in milk, such as MPOx, MP and MT to evaluate their synergistic effect on BuChE. Good linearity was obtained in the range 0.005-50 µg L^{-1} for MPOx and 0.5-1000 µg L^{-1} for MP as well as MT in milk. The developed bioassay shows recovery rates for MPOx in spiked milk samples lay between 93.2%-98.6%. The proposed method can be adopted for rapid screening of milk samples in 384 well plate formats with further miniaturization presented in 1536 well plates. Short analysis time (12 min) and high reproducibility are the key features of the developed bioassay.

Chapter 3 Development of biochip device for analysis of OPs in milk Micro and nano fabrication technologies offer small size chip devise. The biochip has ability to use very small quantities of samples and reagents and to carry out separations and detections with high resolution and sensitivity; low cost; short times for analysis; and small footprints for the analytical devices. In this work, the chip fabrication was done in CARE, IIT Delhi. The miniaturized assay on developed biochip using AChE was successfully demonstrated for screening low level OPs in milk using 5.5 µL assay volume. The study demonstrated one of the

significant attempts for high throughput CL based detection for MPOx in milk using biochip devise wherein up to 64 samples can be analyzed simultaneously. The biochip could be stable at room temperature for more than 3 months without significant loss in enzymatic activity. A major achievement of the work is miniaturization of assay and point of contamination testing at milk collection centres with ease of operations. The developed biochip can determine OPs concentration lower down to 0.001 µg L^{-1} in milk. The present biochip assay has multifold applications in dairy industries and milk collection centers with ease and use of microwell chip device as a screening technology. The biochip based results has been cross validated with standard chromatographical techniques LC-MS/MS and showed good co relation.

Chapter 4 A novel on chip inhibition assay for mercury analysis in water The work demonstrated a novel on chip inhibition assay for Hg^{2+} analysis in water using GOx immobilized on aluminium biochip. The method is simple, economical and rapid to screen Hg^{2+} in water. On chip assay for Hg^{2+} was found highly sensitive with LOD 0.25 µg L^{-1}. The obtained inhibition is purely due to the enzyme immobilized on chip with total assay volume of 5 µL. Assay on chip allows testing for trace metals to be performed more reliably and easily. Further improvements can be done in the assay using more selective enzymes with other HMs.

Chapter 5 A visual colorimetric bioassay for determination of pesticides in drinking water A visual yet sensitive colorimetric assay for OPs is demonstrated in drinking water. The presented work utilizes immobilized ALP on a disposable micro column with the following merits; (a) the technique is affordable and simple to perform and interpret the results visually (b) the assay is optimized to meet the drinking water quality criteria for safe drinking water in fields and (c) ALP coupled micro-silica column as a disposable bio-component for analysis of pesticides in water. In this work, micro sized SiO_2 particles have been successfully employed as sensitive matrix to immobilize ALP enzyme to fabricate a novel visual, disposable column assay. The colorimetric results showed that the sensor could rapidly and sensitively determine OPs and CM under optimized condition with very small assay volume. The assay is free from interferences of tested metal ions at low level. The presented bioassay exhibited a color change, good linear detection range and high sensitivity for OPs. The visually detected analytes are also

quantified using colorimetric well plate assay. Thus, the proposed bioassay can be useful to provide safe drinking water to the common population.

Chapter 6 Novel strategies for automated detection and detoxification of OPs in milk The development of an automated flow-based biosensor test for detection and detoxification of OPs in milk was successfully demonstrated. Biosensor B394 could determine EPOx, chlorpyrifos-oxon (CPO) and malaoxon (MAO) lower down to 5×10^{-9}, 5×10^{-12} and 5×10^{-10} M, respectively in milk. The developed system could successfully determine the presence of OPs in milk. The test could be completed in 15 min with good reproducibility. The proposed system can be applied for online monitoring of OPs in milk processing units and collection centres. Investigation also showed the potential of PTE as a detoxification tool for OPs in water and milk. Three most toxic insecticides were tested to check the cumulative detoxification effect in the presence of PTE. Under optimum conditions, a column containing 335 IU of PTE was shown to immediately detoxify EPOx, MAO and CPO in the down to 1×10^{-9} M. In the case of MAO, the detoxification was lower, due to the very slow hydrolysis of this pesticide by PTE. In case of EPOx and CPO, the detoxification was found 86% and 76%, when the detoxified product was evaluated using flow based biosensor. OPs mixtures were also significantly detoxified in milk up to 86%.

7. References

European Communities, Commission Directive1999/50/EC of 25may 1999 amending Directive 91/321/EEC on infant formulae and follow-on formulae. *Official Journal of the European Communities* 1999, 139, 29.

Istamboulie G., Durbiano R. Fournier D., Marty J. L., Noguer T. Biosensor-controlled degradation of chlorpyrifos and chlorfenvinfos using a phosphotriesterase-based detoxification column. *Chemosphere* 2010, 78: 1-6.

Laetz C. A., Baldwin D. H., Collier T. K., Hebert V., Stark J. D., Scholz N. L. The Synergistic toxicity of pesticides: Implications for risk assessment and the conservation of endangered pacific salmon. *Environmental Health Perspectives*, 2009, 117: 348-353.