Functionalized surfaces for optical biosensors: Applications to *in vitro* pesticide residual analysis

J. SVITEL[1], I. SURUGIU[1], A. DZGOEV[2], K. RAMANATHAN[3], B. DANIELSSON[1]
[1]*Pure and Applied Biochemistry, Lund University, Box 124, 22100 Lund, Sweden*
[2]*Medic House of Scandinavia A/S, Ostre Havnevej 31, DK-5700 Svendborg, Denmark*
[3]*US Environmental Protection Agency, 944 E. Harmon Avenue, Las Vegas, NV 89119, USA*

Functionalized biosensing surfaces were developed for chemiluminescent immunoassay of pesticides. Two approaches to construct functionalized surfaces were tested: (i) pesticide is immobilized to the surface and interacts with a labeled antibody; (ii) antibody is immobilized and interacts with a labeled pesticide. As labels alkaline phosphatase and peroxidase were used with their corresponding substrates CSPD and luminol, respectively. Light produced by chemiluminescent substrate was detected by a thermoelectrically cooled CCD camera or a photomultiplier. The best detection limit 0.00001 ng/ml was obtained using antibodies immobilized to dextran-enhanced surface. Completely renewable surface was obtained using reversible lectin-monosaccharide interaction, one surface was used for 200 analyses without any loss of binding capacity. Most favorable stability and cost per analysis was achieved with molecularly imprinted polymer (MIP) instead of antibody. The functionalized biosensing surfaces were prepared to detect 2,4-dichlorophenoxyacetic (2,4-D) acid as a model pesticide. The developed concepts are, however, generally applicable to other pesticides and to other optical formats, e.g. optical fiber.

1. Introduction

Exposure of humans to pesticides may result in a number of consequences varying from acute to chronic effects. High concentrations of some pesticides, especially organophosphates and carbamates, result in acute effects on the central and peripheral nervous system. Clinical manifestation and mechanism of acute effects of organophosphate, carbamate and pyrethroid insecticides on the central and peripheral nervous systems were reported [1]. Acute poisoning may be followed by persistent effects and the effect of long-term low level pesticide exposure [1]. Long-term low level pesticide exposure can cause a number of effects on the immune system, varying from a slight modulation of immune functions to the development of clinical immune diseases [2]. Especially susceptible to the pesticide exposure are children. A study focused on the immune system of children revealed abnormalities in cytokines associated with hematopoiesis [3]. Additionally, elevations in pro-inflammatory cytokines and neuropeptides indicated a state of generalized and neurogenic inflammation [3]. A group chronically exposed to pesticides and being most at risk from pesticide toxicity are farm workers. Study investigating subclinical morbidity patterns among male farm workers was recently published [4]. The study showed significantly depleted erythrocyte acetylcholin-esterase activity and hemoglobin-adjusted erythrocyte acetylcholinesterase activity in established farm workers in statistically significantly higher numbers than in referent and new farm workers.

Approximately 800 pesticides are currently in use. New methods for pesticide residue monitoring are prevalently based on bio-sensing principles. The methods are based on enzyme-linked and antibody-antigen biorecognition principle. The application of enzyme-linked principles is limited because only a few enzymes interfere with pesticides. The principal advantage of immunochemical methods is that immuno-analyses principally can cover whole range of pesticides. Some immunochemical methods have already been developed using techniques previously used for medical purposes such are RIA, ELISA, and chemiluminescent ELISA. Recently, molecularly imprinted polymers (MIPs), known as ''plastic antibodies'', were reported to replace antibodies and enzymes as recognition elements. MIPs were used in chromatographic method [5] and chemiluminescent ELISA-like assay [6].

Pesticides that have attracted most research interest and are used as model pesticides for various studies and are also most frequently published are 2,4-dichlorophen-oxyacetic acid and triazine herbicides simazine and atrazine. First, studies adopted previously developed RIA and ELISA formats to these pesticides [7–9] . Despite the success of the microtitre plate based ELISA other formats, such as capillary [10], optical fiber [11], microplate [12], membrane strips [13], piezoelectric [14], were also developed. Chemiluminescent ELISA offer the advantages of very sensitive detection and favorable signal to noise ratio [15] and can cover a wide range of agrochemical compounds [16]. Extremely high

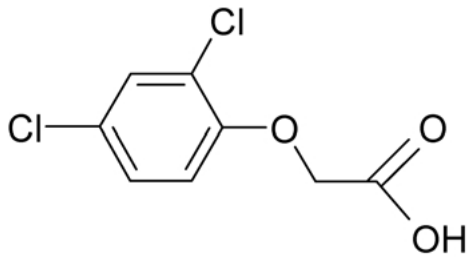

Figure 1 Structure of 2,4-dichlorophenoxyacetic acid (2,4-D).

sensitivity was recently obtained using a thermoelectrically cooled CCD camera coupled to chemiluminescent ELISA [12]. SPR-based detection technique was also used for the determination of simazine [16] and atrazine [17] in water samples. Recently, an immunoassay based on SPR using BSA-2,4-D conjugate was also reported [18].

2,4-dichlorophenoxyacetic acid (2,4-D) was used as a model pesticide in this study (Fig. 1). 2,4-D is systemic herbicide used to control broadleaf weeds and is used on agricultural crop sites, rande and pasture land, lawn and turf. Short-term exposure to 2,4-D at high doses by the oral route results in progressive symptoms of muscular incoordination, hindquarter paralysis, stupor, coma, and death in animals.

All immunochemical techniques mentioned above involve some type of antibody, or other compound, immobilization to create a biosensing surface. The principal goal of the present study was to develop universal functionalized surfaces, generally applicable to a wide range of immunochemical optical biosensors.

2. Materials and methods

2.1. Surface chemistry

Microplates for chemiluminescence ELISA were purchased from Cel-Line (New Field, NJ, USA). The glass surface of microplate was aminosilanized and printed with hydrophobic ink to create a pattern of 4 × 20 square microwell, each 2 × 2 mm. Each microwell can hold up to 5 µl liquid. The surface was activated with 2% glutardialdehyde for 5 min. Then, each microwell was incubated with 2 µl of the 1 µg/ml 2,4-D-BSA conjugate for 1 h at room temperature. Finally, active groups were blocked with 0.2% BSA solution.

The chips used for SPR measurements were carboxylated dextran-coated chips CM5 (BIACore, Sweden). Carboxy groups were activated with solution containing 5.7 mg/ml N-hydroxysuccinimide and 37.5 mg/ml N-ethyl-N′(3-dimethylaminopropyl)carbodiimide for 7 min. Surface was modified with 1 mg/ml *p*-aminophenyl-α-D-glucopyranoside for 10 min. Then the surface was deactivated with 1 M ethanolamine pH 8.5.

Glass capillaries (Sarasota, FL, USA) were cleaned with boiling HNO_3 (10%) for 4 h followed by washing with water. For sol-gel coating about 2.25 ml TEOS, 0.7 ml water and 50 µl of 0.1 M HCl were mixed and sonicated for 3 min. Thereafter, the contents were stirred for 4 h at room temperature, to obtain a clear sol. The sol was diluted with deionized water 1 : 4 of sol : water. This was used for further experiments. Glass capillaries filled with the sol were incubated for 1 h on a shaker to facilitate uniform coating. This was followed by drying of the capillaries for 1 h at room temperature.

Capillaries with dextran-enhanced surface were prepared by covalently binding carboxylated dextran on the aminosilanized glass surface. Antibodies were bound to the dextran carboxylic groups by the method similar to that used for SPR chips (described above).

MIP-modified capillaries were prepared by the polymerization of trimethylolpropane trimethacrylate (crosslinker), 4-vinylpyridine (functional monomer), 2,2′-azobis-isobutyronitrile (polymerization initiator), and 2,4-D (imprint molecule) in capillaries. Complete process was described in details previously [20].

2.2. Antibodies and bioconjugate preparation

Monoclonal anti-2,4-D antibodies from two sources were used: Veterinary Research Institute, Brno, Czech Republic (provided by Dr Franek) and Moscow State University, Moscow, Russia (provided by Dr Eremin). Conjugates of proteine (BSA, ConA, HRP, or TOP) with 2,4-D were prepared using carbodiimide method. 2,4-D was activated in solution containing 20 mM 2,4-D, 40 mM NHS, 80 mM EDC in dimethylformamide for 2 h. 0.5 ml of activated 2,4-D was mixed with 10 mg of protein dissolved in 1 ml water and let to react for 1 h at RT and 16 h at 5 °C. Then conjugates were purified using dialysis.

2.3. Measurement techniques

Each microwell of modified microplate for chemiluminiscence ELISA was incubated with antibody preincubated with different concentrations of 2,4-D, then incubated with AP labeled antimouse Ab and finally CSPD-Emerald II enhancer (Tropix Inc., Bedford, MA, USA) mixture was added to each microwell. The plate was imaged in a dark chamber (Fig. 2b) with the Photometrix 200 CCD camera (Photometrix, Tucson, AZ, USA) thermoelectrically cooled at 40 °C (exposure time 90 s).

The SPR measurements were performed with BIACore 2000 (BIACore, Sweden) using HEPES buffer. The measurements were performed by sequential injections of 0.5 mg/ml ConA-2,4-D cojugate, 0.1 mg/ml anti-2,4-D antibody preincubated with 2,4-D standards (1–400 ng/ml), and 1 mg/ml *p*-aminophenyl-α-D-glucose (regeneration solution).

A photomultiplier PMT Sensor Module HC135-02 (Hamamatsu, USA) connected to light–tight capillary holder by a optical fiber bundle (Fig. 2a) was used for capillary chemiluminescent assay. Peroxidase conjugates (TOP or HRP) were mixed with 2,4-D standard solutions, injected to capillaries and incubated for 15 min. Then capillaries were filled with luminol-enhancer mixture and emitted light was measured during 1 min period.

3. Results and Discussion

Various surface chemistries were used and compared in this work. The principles of binding of interacting molecules are summarized in Fig. 1. As can be seen

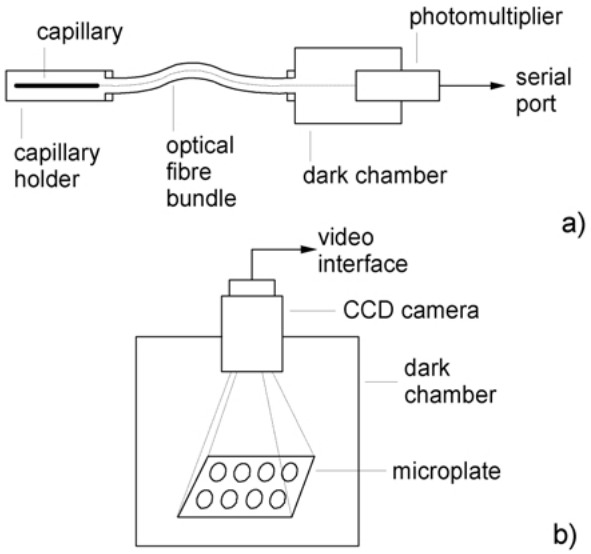

Figure 2 Instrumental set-ups used for pesticide analysis with functionalized surfaces: photomultiplier equipped with capillary holder (a); CCD camera adapted for microplate reading (b).

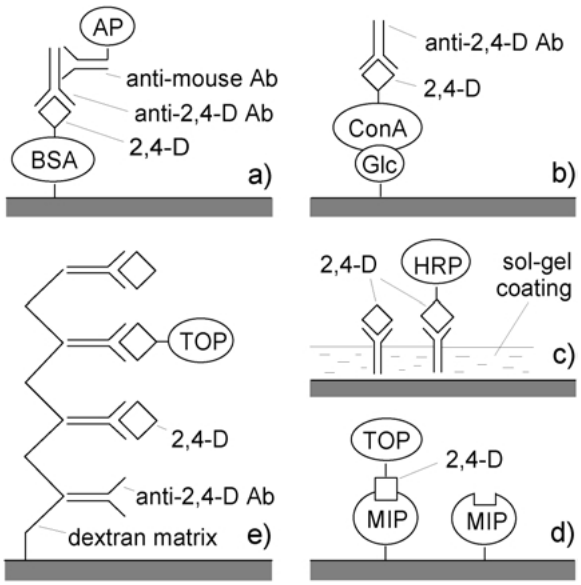

Figure 3 The architecture of functionalized surfaces: chemiluminescence imaging ELISA (a); surface plasmon resonance (b); chemiluminescence capillary assay (c); use of MIPs instead of antibodies (d); surface binding capacity enhanced by dextran matrix (e). Abbreviations: bovine serum albumin (BSA); alkaline phosphatase (AP); Concanavalin A (ConA); glucose (Glc); horseradish peroxidase (HRP); tobacco peroxidase (TOP); molecularly imprinted polymer (MIP).

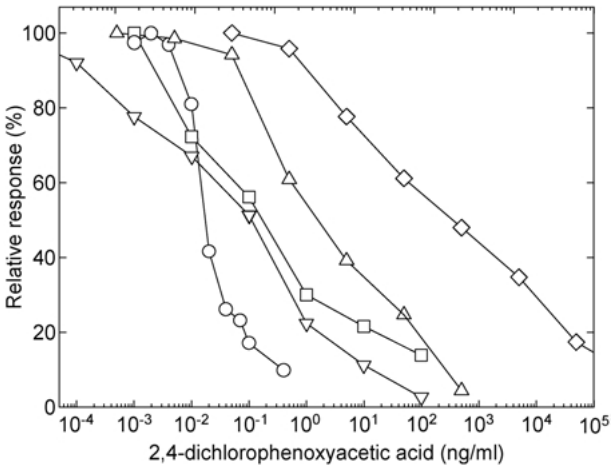

Figure 4 Comparison of calibration curves for 2,4-dichlorophenoxyacetic acid determination using various functionalised surfaces: chemiluminescence imaging ELISA (□); surface plasmon resonance/ (○); chemiluminescence capillary assay (△); use of MIPs instead of antibodies (◇); surface binding capacity enhanced by dextran matrix (▽).

from Fig. 1, two approaches are available to develop a pesticide immunoassay: (i) immobilized pesticide (Fig. 3a,b) or (ii) immobilized antibody (Fig. 3c–e). While one interacting molecule is immobilized to the surface another interacting molecule, which is free in solution, must be labeled with light producing enzyme. Actually two light producing enzymes are generally used: horseradish peroxidase (HRP) using luminol as a substrate and alkaline phosphatase using CSPD as a substrate.

Chemiluminescent immunochemical assay of 2,4-D was developed using glass microplates functionalized with pesticide. Functionalized surface was obtained by pesticide immobilization. For that purpose conjugate BSA-2,4-D was prepared and protein moiety (BSA) was covalently bound on the microplate surface (Fig. 3a). Chemiluminescence (light) was detected using a charge coupled device (CCD) camera. Standard CCD sensors exhibit high background current, and thus unfavorable detection limit, at room temperature. For that reason a thermoelectrically cooled CCD camera was used and after optimization very low detection limit 0.001 ng/ml was achieved. A surface functionalized with covalently bound pesticide is supposed to be disposable (for one use only). We tried to develop renewable functionalized surface. To achieve regeneration possibility we used lectin-monosaccharide interaction to immobilize pesticide to the surface (Fig. 3b). Interaction between ConcanavalinA (lectin) and α-D-glucose (monosaccharide) is completely reversible. Thus, surface regeneration can be achieved using excess of monosaccharide. This concept was tested using SPR technique, one surface was used and regenerated up to 200 times without any loss of binding capacity. Furthermore, current SPR instrumentation enables automated operation and multi-pesticide assay.

Capillary format was tested for the approach using immobilized antibody. This format is especially suitable for construction of portable devices. Light detection was performed by photomultiplier (PMT). Current state-of-the-art PMTs are relatively cheap and sensitive. They are capable of detection of individual photons. Anti-pesticide antibodies were immobilized in silicate gel on inner capillary wall (Fig. 3c). This approach led to a very good detection limit. The detection limit was further improved by enhancing surface binding capacity using carboxylated dextran matrix. Dextran contains high portion of functional groups that are suitable (after activation) to bind antibodies and avoid steric hindrance for further antibody interaction (Fig. 3e). Furthermore, tobacco peroxidase which provide more efficient chemiluminescent reaction was tested. This approach led to extremely low detection limit 0.00001 ng/ml. Another method to prepare functionalized surfaces was to use molecularly imprinted polymer (MIP), so called ''plastic antibodies'', instead of antibody. We obtained

TABLE I Detection techniques used for residual pesticide detection

Functionalized surface	Detection technique/applicable assay format	Applicable range (ng/ml)
Pesticide conjugate covalently bound to surface (Fig. 3a)	Light detection by CCD camera/microplates	0.001–10
Antibody covalently bound to surface	Light detection by CCD camera/capillary	0.1–100
Lectin bound to sugar-modified surface (renewable) (Fig.3b)	Surface plasmon resonance	3–100
Antibody immobilized in sol-gel (Fig 3c)	Light detection by photomultiplier/capillary, optical fiber	0.05–500
MIP modified surface (Fig. 3d)	Light detection by photomultiplier/capillary	0.5- – 10^6
Dextran-enhanced surface (Fig. 3e)	Light detection by photomultiplier/capillary (microplates)	0.00001–0.1

lower detection limit, however the use of MIPs provides extremal stability and favorable cost per analysis. The calibration curves obtained with different functionalized surfaces described above are presented in Fig. 4. As can be seen from Fig. 4, various methods used to functionalize surfaces resulted in a wide range of detection limits and also in slight variability in calibration curve slopes. Based on the presented curves the concentration ranges suitable for practical assays are summarized in Table I.

4. Conclusion

The surface modifications presented here were tested with one model pesticide, however they are also applicable to other pesticides. Adaptation of 2,4-D assay to another pesticide involves raising another specific antibody, either monoclonal or polyclonal, and synthesis of another complementary pesticide-enzyme label conjugate. Using fluorescent labeled pesticides is also possible, however according to our literary survey lower detection limits should be expected. We also believe that the concepts presented here are of general applicability and can be extended to other optical formats, e.g. microplates, optical fiber.

Acknowledgment

J.S. acknowledges support from EC (Marie Curie Fellowship QLK1-CT-1999-51347).

References

1. F. S. HE, *Neurotoxicology* **21** (2000) 829.
2. C. COLOSIO, E. CORSINI, W. BARCELLINI and M. MARONI, *Toxicol. Lett.* **108** (1999) 285.
3. T. M. PHILLIPS, *J. Exp. Anal. Env. Epidemiol.* **10** (2000) 769.
4. J. GOMES, O. LLOYD, M. D. REVITT and M. BASHA, *Scand. J. Work Env. & Health.* **24** (1998) 213.
5. E. HOGENDOORN and P. ZOOEN, *J. Chrom. A* **892** (2000) 435.
6. I. SURUGIU, B. DANIELSSON, L. YE, K. MOSBACH and K. HAUPT, *Anal. Chem.* **73** (2001) 487.
7. D. F. RINDER and J. R. FLEEKER, *Bull. Environ. Contam. Toxicol.* **26** (1981) 27.
8. D. KNOPP, P. NUHN and H. DOBBERKAN, *Arch. Toxicol.* **58** (1985) 27.
9. A. N. ZHERDEV, B. B. DZANTIEV and J. N. TRUBACEVA, *Anal. Chim. Acta* **237** (1997) 131.
10. B. DANIELSSON, I. SURUGIU, A. DZGOEV, M. MECKLENBURG and K. RAMANATHAN, *Anal. Chim. Acta.* **426** (2001) 227.
11. Z. P. CHEN, D. L. KAPLAN, H. GAO, J. KUMAR, K. A. MARX and S.S. K. TRIPATHY, *Mat. Sci. & Eng. C* **4** (1996) 155.
12. A. DZGOEV, M. MECKLENBURG, P.-O. LARSSON and B. DANIELSSON, *Anal. Chem.* **68** (1996) 3364.
13. M. Y. RUBTSOVA, J. V. SAMSONOVA, A. M. EGOROV and R. D. SCHMID, *Food. Agric. Immunol.* **10** (1998) 223.
14. M. MINUNNI, P. SKLADAL and M. MASCINI, *Anal. Lett.* **27** (1994) 1475.
15. A. DZGOEV, I. G. GAZARYAN, L. M. LAGRIMINI, K. RAMANATHAN and B. DANIELSSON, *Anal. Chem.* **71** (1999) 5258.
16. A. M. JIMENEZ and M. J. NAVAS, *Crit. Rev. Anal. Chem.* **27** (1997) 291.
17. C. MOUVET, R. D. HARRIS, C. MACIAG, B. J. LUFF, S. J. WILKINSON, J. PIEHLER, A. BRECHT, G. GAUGLITZ, R. ABUKNESHA and G. ISMAIL, *Anal. Chim. Acta.* **338** (1997) 109.
18. M. MINUNNI and M. MASCINI, *Anal. Lett.* **26** (1993) 1441.
19. R. P. REVOLTELLA, L. L. ROBBIO and B. LIEDBERG, *Biotheraphy* **11** (1998) 135.
20. O. BRUEGGEMAN, R. FREITAG, M. WHITCOMBE and E. VULFSON, *J. Chromatogr. A* **781** (1997) 43.

Received 14 May
and accepted 30 May 2001