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# Biosensor-Assisted Selection of Optimal Parameters for Designing Molecularly Imprinted Polymers Selective to Phosmet Insecticide

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## Abstract

Molecularly imprinted polymers (MIPs) for phosmet insecticide were synthesized by batch polymerization. The affinity of functional monomers to phosmet was tested using an original method involving an electrochemical biosensor based on acetylcholinesterase inhibition. It was demonstrated that association of phosmet with appropriate functional monomers resulted in a decrease of enzyme inhibition. Using this method, it was shown that N,N-methylenebis(acrylamide) displayed the highest interactions with phosmet using DMSO as solvent. These results were in good accordance with those obtained by conventional computational modeling. Molecularly imprinted polymers (MIPs) and non-imprinted polymers (NIPs) were synthesized and adsorption isotherms were studied to describe their interaction with phosmet. Freundlich isotherm was able to fit phosmet adsorption on MIPs with good agreement ($R^2$ = 0.9). The pre-exponential factor $K_F$ determined for MIPs was 1.439 $mg^{(1-N)}.g^{-1}.L^N$, more that 10 times higher than for NIPs (0.125 $mg^{(1-N)}.g^{-1}.L^N$, indicating an increase of binding sites number and average affinity.

Graphical abstract

Keywords

phosmet; molecularly imprinted polymers; biosensor; monomer selection

## 1. Introduction

Phosmet is an organophosphorus insecticide (OP) widely used in olive culture as a preventive treatment against the olive fruit fly *Dacus oleae,* and also in apples, grapes, peaches, potatoes and prunes culture [(1)]. As all OP, its presence in food presents a major risk for consumers health, and its residues are regulated through several organisms [(2,3)]. The first step of OP determination involves their extraction from food matrix. A traditional approach to extract and isolate phosmet residues from food is based on liquid-liquid extraction [(4,5)], which is usually followed by a clean-up step, such as solid-phase extraction [(6)] or chromatographic purification before analysis [(7)]. Despite the quality of separation of pesticide residues from the matrix, these procedures are time-consuming, laborious, and often require large amounts of potentially hazardous solvents [(8)]. In order to minimize solvent volumes and to combine extraction and clean-up into a single step, several innovative approaches have been described such as solid-phase micro-extraction (SPME) [(9)], matrix solid-phase dispersion (MSPD) [(10)], and molecularly imprinted solid phase extraction (MISPE) [(11)].

Molecular imprinting technology allows designing artificial receptors with specific cavities in polymer matrices [(12)]. Molecularly imprinted polymers (MIPs) are obtained by copolymerization of a cross-linker and a functional monomer (FM) in the presence of a template molecule. To achieve this, FM must be pre-arranged around a template molecule in a

porogenic solvent. Therefore, the most important challenge in MIPs synthesis is to find the suitable FM that can exchange the highest interactions with the template, forming stable complexes in order to achieve high selectivity and rebinding capacity [13]. Essentially, two molecular imprinting approaches have been described, based on reversible covalent bonds or non-covalent interactions between template and FM. Regarding the limited number of potential templates suitable with the first approach, the non-covalent approach is the most frequently used method to prepare MIPs due to its simplicity [14]. In this approach the template interacts with FM by non-covalent bonds (hydrogen bonds, electrostatic interactions, Van der Waals bonds) [15].

Over the past few years, many strategies have been reported for the selection of optimal FM, including computer simulation, spectroscopic measurements [16] and isothermal titration calorimetry [17]. In this work, we present for the first time the possibility of using a biosensor-based method for selecting the more appropriate FM and porogenic solvent.

The primary toxicity of OP pesticides, including phosmet, comes from the irreversible inhibition of the enzyme acetylcholinesterase (AChE, EC 3.1.1.7), resulting from the phosphorylation of its active-site serine [18]. The entrance of AChE active site is controlled by a lot of side chains lining the active-site gorge, which come into contact with the insecticide before reaching the catalytic site [19]. Recently our team has described a biosensor for phosmet detection based on the use of a genetically-engineered drosophila AChE [20]. In this enzyme, mutations of active site gorge amino acids were made, allowing a highly sensitive detection of organophosphorus insecticides [21]. In the present work, it was postulated that the formation of phosmet/FM complex could have an effect on AChE inhibition, thus allowing an estimation of resulting interactions.

One of the best methods for evaluating binding capacity and selectivity is the batch rebinding [(12)], which allows comparing MIPs and NIPs (non-imprinted polymers) molecular recognition, expressed as the binding capacities or imprinting factors. Langmuir and Freundlich isotherms represent suitable theoretical models for characterizing and understanding binding affinity on MIPs. These methods, which are also called continuous heterogeneous binding models, can well characterize MIPs by calculating the total number of binding sites, heterogeneity index and mean binding affinity. Among them, the Freundlich isotherm is the most commonly model used for characterizing MIPs [(22)].

In this work, three functional monomers were pre-selected using computer simulation and the effect of each phosmet/FM complex on AChE-based sensor response was studied using 3 different porogenic solvents. New MIPs for phosmet were therefore synthesized in selected conditions and their adsorption capacity was compared with NIPs. Finally, the adsorption kinetics of MIPs and NIP were studied to validate the presence of receptor cavities and the affinity distribution.

## 2. Materials and methods

### 2.1. Reagents

Phosmet PESTANAL® (2-(dimethoxyphosphinothioylthiomethyl) isoindoline-1,3-dione), acrylamide (ACR), N,N-methylenebis(acrylamide) (MBAA), itaconic acid (IA), ethylene glycol dimethylacrylate (EGDMA) acetylthiocholine chloride (ATCh), N-bromosuccinimide (NBS), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB-Ellman's reagent), AIBN (2,2′-azobis(2-methylpropionitrile)), acetonitrile HPLC grade (ACN), N,N-dimethylformamide for HPLC (DMF) and dimethyl sulfoxide anhydrous (DMSO) were purchased from Sigma-Aldrich (France).

Genetically-modified AChE from *Drosophila melanogaster* type B394 was produced by Protein Bio Sensor (PBS, Toulouse, France). Graphite (Electrodag 423 SS) and silver/silver chloride (Electrodag 418 SS) inks were obtained from Acheson (Plymouth, UK). Cobalt phtalocyanine-modified carbon paste was purchased from Gwent Electronic Materials, Ltd. (Gwent, UK). Poly(vinyl)chloride (PVC) sheets (200 mm × 100 mm × 0.5 mm), supplied by SKK (Denzlingen, Germany), were used as support for the screen-printed electrodes. A glycerophthalic paint (Astral, France) was used as insulating layer. The photocrosslinkable poly(vinyl alcohol) polymer PVA-AWP (Toyo Gosei, Japan) was used for enzyme immobilization.

**2.2. Molecular modeling of template/monomer interactions**

Computational analysis was performed at the University of Leicester using the ALICE supercomputer running the SPECTRE operating system. Phosmet/FM interactions were simulated using the SYBYL 7.3 (Tripos Inc. ST. Louis, MO, USA) modeling software package, in conjunction with the Leapfrog ™ algorithm [(11)]. A molecular model of phosmet was created and charged with the Gasteiger-Huckel computational method, prior to energy minimization. An optimized structure was generated by minimization of the charged template over 15,000 iterations to a value of 0.001 kcal $mol^{-1}$. Simulated dynamics experiments were performed by applying the rapid heating and cooling to the phosmet molecule to ensure conformational freedom of all bonds [(23)]. In order to achieve the most energetically favorable structure, the simulation was carried over a temperature range of 700-300 K for 1,000,000 iterations at each temperature.

**2. 3. Determination of Acetylcholinesterase activity**

Spectrophotometric measurements were performed using a Hewlett Packard diode array

8451A spectrophotometer. The AChE activities were measured at 412 nm in 0.1 M phosphate buffer pH 7 using 1 mM of substrate (ATCh) and 0.03 M DTNB according to the procedure described by Ellman et al [24].

### 2.4. Biosensor construction

Screen-printed electrodes were fabricated using a semi-automatic DEK248 printing machine according to a procedure previously described [25], in a three-electrodes configuration. The working electrode incorporating cobalt phtalocyanine, as mediator was a 4 mm-diameter disk, the auxiliary electrode was a 16 mm x 1.5 mm curved line and the Ag/AgCl pseudo-reference electrode was a 5 mm x 1.5 mm straight track. The polymer PVA-AWP was mixed with enzymatic solution in a ratio 70:30% (v/v). The mixture was vortex-mixed and a volume of 3 µL was spread onto the cobalt phtalocyanine modified working electrode. The initial concentration of AChE solution was adjusted in order to obtain a final enzyme loading of 1 mU/biosensor. The electrodes were then exposed for 3 h under a neon lamp (15 W) at 4 °C to carry out photopolymerization, they were ready to use after 48 h drying at 4 °C [20].

### 2.5. Amperometric measurements

The activity of AChE immobilized on the electrodes was determined by electrochemical oxidation of thiocholine formed upon enzymatic hydrolysis of ATCh. Amperometric measurements were carried out using a 641VA potentiostat (Metrohm, Switzerland), connected to a BD40 flatbed recorder (Kipp & Zonen, The Netherlands). As described in previous works, the applied potential was 100 mV *vs.* Ag/AgCl [26]. In order to make amperometric measurements, the biosensor strip was vertically inserted into an analytical cell containing 10 mL of measurement buffer (phosphate buffer 0.1 M, pH 7, containing KCl 0.1

M and NBS $10^{-6}$ M). The effect of phosmet on AChE based biosensor was studied in a three-step procedure as follows. The sensor initial response was first measured in triplicate ($A_0$) in 10 mL of measurement buffer containing 1 mM ATCh. After rinsing, the electrode was incubated for 10 min with 10 µL of inhibitor in 10 mL of buffer and the residual response (A) was finally recorded. The percentage of inhibition was calculated according to the following formula:

$$I(\%)= \{(A_0-A)/ A_0\}*100$$

**2.6. Preparation of phosmet/functional monomer (FM) mixtures**

Phosmet solutions were prepared in different solvents (ACN, DMF or DMSO) and used to establish the calibration curves for phosmet detection. Mixtures of phosmet and different FM were also prepared in each solvent with a molar ratio of 1:4. The mixtures were incubated at different temperatures, for various periods of time, re-diluted in the same solvent and injected in the measurement cell. The final concentration of phosmet in the cell was $10^{-8}$ M. In the absence of phosmet, the effect on biosensor response of the pre-selected monomers diluted in each solvent was studied in the same conditions.

**2.7. HPLC determination of phosmet**

The quantification of phosmet was performed using an HPLC L-2000 series LaChrom Elite® system from Merck-Hitachi (VWR, France). Chromatographic separations were carried out with a C18 Luna® reverse phase column (250 × 4.6 mm, 5µm) using acetonitrile/water mixture (65/35, v/v) as a mobile phase at flow rate 1 $mL.min^{-1}$. The injection volume was 20 µL. Phosmet was detected at 228 nm and data acquisition was performed using EZchrom Elite software. The quantification of phosmet was conducted by measuring the peak area and comparing it with the relevant calibration curve. The linear regression of calibration curve

was $y(area)=1.665x10^{11}(C_{phosmet(M)})+ 9.24x10^{4}$ with a dynamic range from $2x10^{-7}$ M to $2x10^{-4}$ M, LOD=$5.10^{-8}$ M and $R^2$=0.99.

### 2.8. Synthesis of imprinted and non-imprinted polymers

Imprinted and non-imprinted polymers were synthesized using the selected functional monomer in the selected porogenic solvent according to biosensor studies. Concerning MIP synthesis, an amount of $0.162x10^{-3}$ mole of phosmet, $0.648x10^{-3}$ mole of functional monomer and $4.24x10^{-3}$ mole of crosslinker (EGDMA) (molar ratio 1:4:26) were dissolved in 5 mL of porogenic solvent and $0.085x10^{-3}$ mole of AIBN as initiator were added to this mixture. The mixture was de-aerated with $N_2$ for 10 min, sealed in a glass bottle and thermally polymerized in oil bath at 60°C for 20 h.

After synthesis, the polymer particles were collected, washed intensively using Soxhlet extraction with ethyl acetate/methanol/acetic acid solution (4:4:2, v/v) for 48 h, then with methanol/acetic acid (8:2, v/v) for 24 h to remove template molecule from the sorbent. HPLC analysis was used to evaluate template removal. Finally, MIPs were oven-dried at 60°C for 24 h. Corresponding non-imprinted polymers (NIPs) were prepared using the same protocol in the absence of template.



### 2.9. Adsorption and kinetics studies

10 mg of imprinted (or non-imprinted) polymers were suspended in 20 mL of (50:50,v/v, ethanol/hexane) solutions containing from $5x10^{-6}$ M to$10^{-3}$ M of phosmet. The mixture was magnetically stirred continuously for 2, 4, 8, 10 and 24 h at room temperature. After

removing polymer particles using syringe filter (0.45 µm PTFE), the concentration of phosmet remaining in solution was evaluated by HPLC analysis.

The equilibrium binding capacities of MIP and NIP sorbents were determined using the following Eq. (1):

$$Qe = (C_i - C_f) \times V/m \quad (1),$$

where $C_i$ and $C_f$ are respectively the initial and final concentrations of phosmet ($mg \cdot L^{-1}$), $V$ (L) is the volume of incubation solvent, and $m$ (g) is the mass of the sorbent.

The Langmuir isotherm model is predicated on the postulation that the structure of the adsorbent is homogeneous, where all sorption sites are similar and energetically equivalent. The general Langmuir equation is given as: $1/ Q_e = 1/ (Q_{max} K_L C_f) + 1/ Q_{max}$,

where $Q_e$ and $Q_{max}$ ($mg.g^{-1}$) are the amounts of sorbate bound per unit mass of sorbent, respectively at equilibrium and saturation, $K_L$ affinity adsorption factor ($L.mg^{-1}$) is the Langmuir constant, $C_f$ ($mg.L^{-1}$) is the equilibrium concentration of sorbate.

The Freundlich isotherm model is most frequently employed to describe sorption onto heterogeneous surfaces from aqueous solution and reversible adsorption, which is not restricted to monolayer formations. It can be expressed in linear form by the following equation: $\text{Log } Q_e = \text{Log } K_F + \text{N Log } C_f$, where $K_F$ and N are the Freundlich constants.

## 3. Results and discussion

### 3.1. Pre-selection of functional monomers by computational modeling

The Leapfrog algorithm was used to screen a database of 28 functional monomers (FM) against the template (phosmet), and allowed analyzing all possible points of molecular interactions between these functional monomers and phosmet. The program was applied for 60,000 iterations and the results were interpreted in terms of empirical binding energies. The Leapfrog program accounts for the various steric contributions, ionic and hydrogen bonds and dipole-dipole interactions, which are expressed as the binding energy. A ranked list of functional monomers was generated with their respective binding energies for the template (Table 1). Selection of the most appropriate monomers, capable of forming the strongest molecular complexes with phosmet, allowed further computational interpretation to be carried out, specific for the intended application.

*Table 1: Simulated binding energies between phosmet (template molecule) and different functional monomers (FM) (see additional information Fig S1)*

| Functional monomers (FM) | Binding energy (kcal $mol^{-1}$) |
|---|---|
| N,N'-Methylenebisacrylamide (MBAA) | -27.7 |
| Acrylamide (ACR) | -26.6 |
| Itaconic acid (IA) | -18.2 |
| Ethylene glycol methacrylate phosphate (EGMP) | -17.8 |
| Methacrylic acid (MAA) | -15.3 |
| 2-(Trifluoromethyl)acrylic acid (TFMAA) | -15.1 |
| Hydroxyethyl methacrylate (HEM) | -13.4 |
| Diethylaminoethyl methacrylate (DEAEM) | -7.4 |
| Ethylene glycol dimethylacrylate (EGDMA) | -5.9 |
| 1,3-Divinylbenzene (mDVB) | -5.8 |
| 1,4-Divinylbenzene (pDVB) | -5.2 |

According to the results of computational modeling (Table 1), MBAA was identified as the most promising monomer, which could form strongest interactions with phosmet (binding energy is -27.7 kcal $mol^{-1}$). Other functional monomers, including ACR and IA, also demonstrated high binding towards phosmet (Fig. 1).

Figure 1

The best three monomers (MBAA, ACR and IA) were thus selected to analyze their affinity towards phosmet using an AChE-based biosensor. In order to study the phosmet/monomer interaction three aprotic solvents able to dissolve these monomers and commonly used as porogenic solvents for MIPs synthesis were tested: acetonitrile (ACN) (polarity index 5.8), N,N-dimethylformamide (DMF) (polarity index 6.4), and dimethylsulfoxide (DMSO) (polarity index 7.2).

### 3.2. Selection of functional monomers using biosensor

#### 3.2.1. Analytical performance of AChE-based biosensor and effect of phosmet

The sensor operational stability was evaluated in order to ensure that signal decrease during inhibition measurements is due to enzyme inactivation by the insecticide and not to enzyme leaking or inactivation by NBS contained in the buffer. The sensor response was shown to be stable for at least 20 successive measurements. The effect of the three selected monomers (MBAA, ACR or IA) diluted in each solvent (ACN, DMF or DMSO) on biosensor response was also tested. It was found that pre-selected monomers and porogenic solvents did not have any denaturing or inhibiting effect on AChE in assay conditions.

The effect of phosmet on AChE based biosensor was studied at concentrations ranging from $2x10^{-10}$ M to $10^{-8}$ M in ACN (Fig. 2). The effect of solvent was also evaluated by testing several phosmet solutions prepared in DMF and DMSO at similar concentrations. As shown in figure 2, the semi-log representation displays a linear response with a correlation coefficient $R^2 = 0.99$ (n = 3) in the studied concentration range. The developed biosensor

showed a LOD of $2x10^{-10}$ M, corresponding to $2x10^{-8}$ M in injected solution. Moreover, it was shown that phosmet induced the same inhibition whatever the solvent used.

Figure 2

### 3.2.2. Effect of phosmet-monomer putative complex on biosensor response

Phosmet (0.032 M) and monomer (0.128 M) were pre-incubated during 30 min at 20 °C and the effect of the resulting complex was studied on AChE-based sensor. Figure 3 presents the decrease of inhibition rate obtained using each functional monomer in three different solvents, the final phosmet concentration being $10^{-8}$ M.

Figure 3

It was observed that, whatever the functional monomer used, a substantial decrease in AChE biosensor inhibition was observed using DMSO as solvent. However, the highest decrease was obtained using MBAA as functional monomer, suggesting that phosmet-MBAA interaction was strong enough to prevent phosmet binding on AChE active site.

These observations were in good agreement with the results obtained upon computational modeling of monomer-template interactions (Table 1).



### 3.2.3. Effect of pre-incubation time on enzyme inhibition

The effect of incubation time on phosmet-monomer interaction was studied for all the pre-selected monomers in DMSO using AChE-based sensor. As shown in figure 4, the association of phosmet and monomers was relatively fast, and no change in inhibition rate was observed

after 30 minutes of contact. Among the three monomers tested, MBAA showed the strongest interaction with phosmet, leading to a 30% decrease of AChE inhibition.

Figure 4

As MIPs synthesis is carried out at 60°C, it was verified that such a temperature has no effect on the formation of template/monomer complex. Phosmet and MBAA were thus pre-incubated for 30 min at 60°C, and the resulting mixture was tested using the biosensor. It was shown that the resulting inhibition was similar to the one observed at 20°C, demonstrating that phosmet-MBAA complex was not destroyed during polymerization.

Imprinted and non-imprinted polymers were therefore synthesized using DMSO as porogenic solvent and MBAA as functional monomer. For MIPs synthesis, MBAA was pre-incubated with phosmet during 30 minutes prior to polymerization.

### 3.3. Adsorption kinetic studies and MIPs validation

MIP and NIP binding kinetics were carried out in presence of phosmet at $5x10^{-4}$ M. As shown in Figure 5a, an optimal binding capacity for MIP was achieved after 10 hours of contact. Binding capacities for MIP and NIP sorbents were thus measured in these equilibrium conditions in presence of different concentrations of phosmet (Figure 5b). The observed difference in binding capacities of MIP and NIP particles can be easily attributed to the molecular imprinting effect.

Figure 5

Adsorption isotherms were studied to describe the interactive behavior between the sorbate (phosmet) and the sorbent (polymer). The applicability of Langmuir and Freundlich isotherm models was studied by judging the correlation coefficients values obtained for each model (Table 2).

*Table 2: Langmuir and Freundlich constants values obtained for MIP and NIP. Q and $C_f$ are respectively expressed in $mg.g^{-1}$ and $mg.L^{-1}$.*

| | Langmuir: $1/Q_e = 1/(Q_{max}\ K_L\ C_f) + 1/Q_{max}$ | | | Freundlich: $\log Q_e = \log K_F + N \log C_f$ | | | |
|---|---|---|---|---|---|---|---|
| NIP | $1/Q_{max}$ | 0.08 | $R^2$=0.94 | Log $K_F$ | -0.903 | $K_F$ = 0.125 | $R^2$=0.78 |
| | $1/(Q_{max}\ K_L)$ | 3.84 | | N | 0.978 | | |
| MIP | $1/Q_{max}$ | -0.004 | $R^2$=0.99 | Log $K_F$ | 0.158 | $K_F$ = 1.439 | $R^2$=0.90 |
| | $1/(Q_{max}\ K_L)$ | 3.19 | | N | 0.698 | | |

As shown is table 2, Langmuir isotherm was able to model phosmet adsorption on NIPs, but not on MIPs, regarding the negative value obtained for the maximum adsorption capacity $Q_{max}$. Generally, the assumption of this model is that the adsorption energy is constant on all sites and that all sorption sites are uniform [27], which is consistent with phosmet adsorption on NIPs. The homogeneity of phosmet adsorption on NIP was also confirmed with Freundlich isotherm model (N ≈1). Freundlich isotherm is applicable to both monolayer and multilayer adsorption, it is based on the assumption of heterogeneous surface energies and has been shown to be generally applicable to most non-covalently imprinted polymers [28]. Freundlich isotherm was able to fit phosmet adsorption on MIPs with good agreement ($R^2$ = 0.9). The pre-exponential factor determined for MIPs $K_F$ = 1.439 $mg^{(1-N)}.g^{-1}.L^N$ was about 10 times higher than for NIPs (0.125 $mg^{(1-N)}.g^{-1}.L^N$), indicating an increase of binding sites number and

average affinity. The value of the second fitting parameter N= 0.698 was lower for MIPs, confirming heterogeneous binding of phosmet.

## Conclusion

This work shows for the first time the possibility of selecting functional monomers appropriate to MIPs synthesis using an enzymatic biosensor. Using DMSO as solvent, it was shown that phosmet-induced inhibition of AChE was decreased in presence of IA, ACR, and MBAA. These results were in accordance with those obtained by modeling molecular interactions, which showed that MBAA was the monomer that shared the strongest interactions with phosmet. The present study opens new opportunities for selecting appropriate monomers devoted to MIPs development, not only for acetylcholinesterase-inhibitors (insecticides, war gases), but also for other enzyme inhibitors like herbicides, fungicides, drugs and toxins.

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Figure caption

*Figure 1. Predicted interactions of Phosmet with a) ACR, b) MBAA and c) IA.*

*Figure 2. Inhibition of AChE-based biosensor as a function of phosmet concentration and solvent*

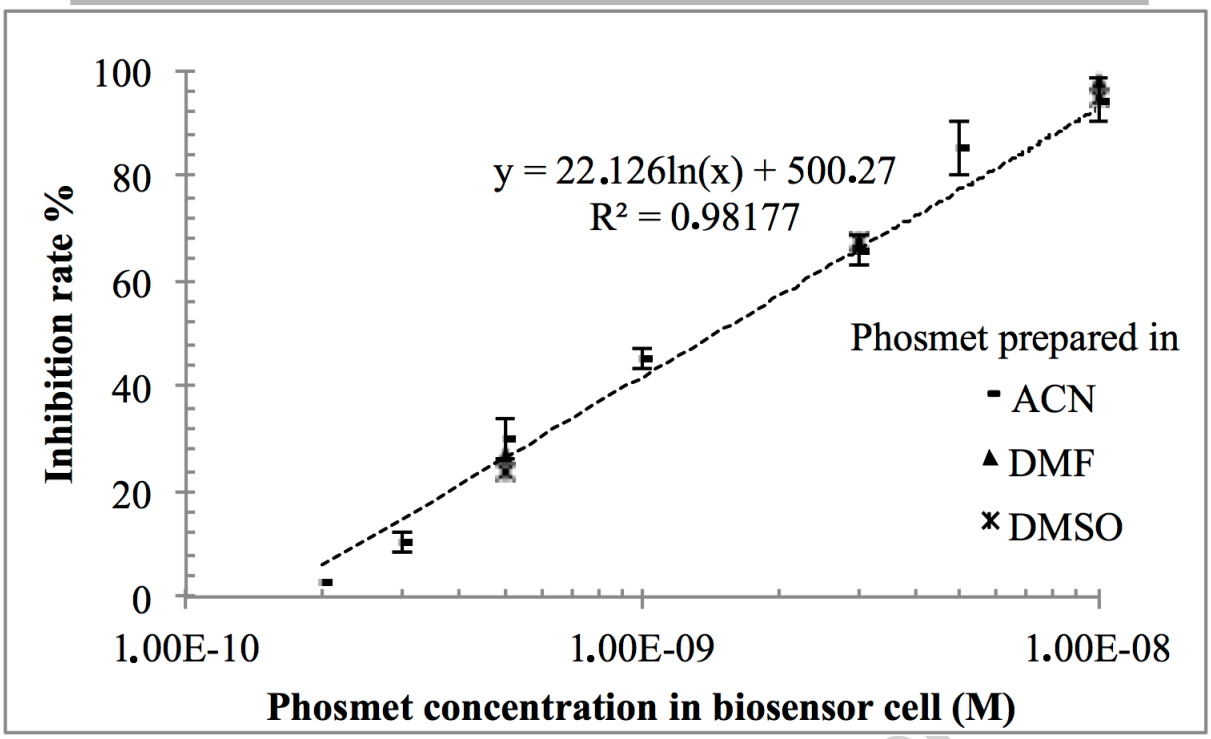
*Figure 3. Inhibition of AChE-based biosensor by phosmet, combined effect of functional monomer (MBAA, ACR, IA) and porogenic solvent (ACN, DMF, DMSO). Results are expressed as percentage of inhibition decrease.*

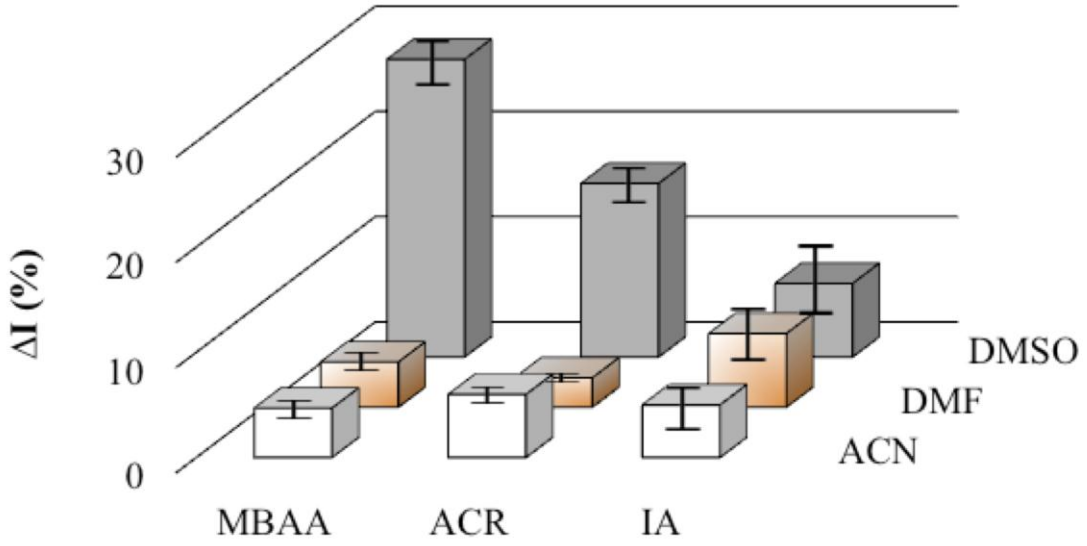
*Figure 4. Effect of pre-incubation time between phosmet and functional monomers (MBAA, ACR, or IA) on biosensor inhibition. Results are expressed as percentage of inhibition decrease.*

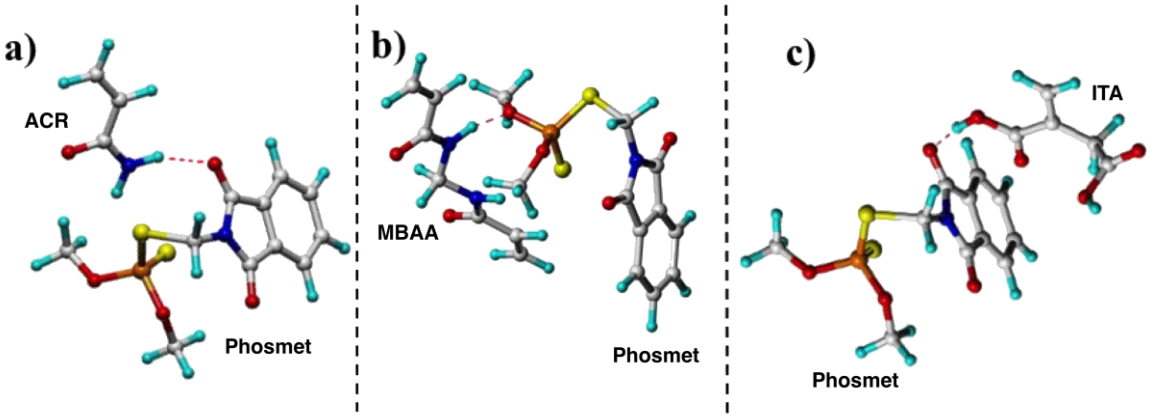
*Figure 5: a) Binding kinetics of polymers (MIP and NIP) in presence of phosmet at $5x10^{-4}$ M (142 mg.$L^{-1}$). b) Binding isotherms obtained for MIP and NIP at equilibrium conditions (10h).*

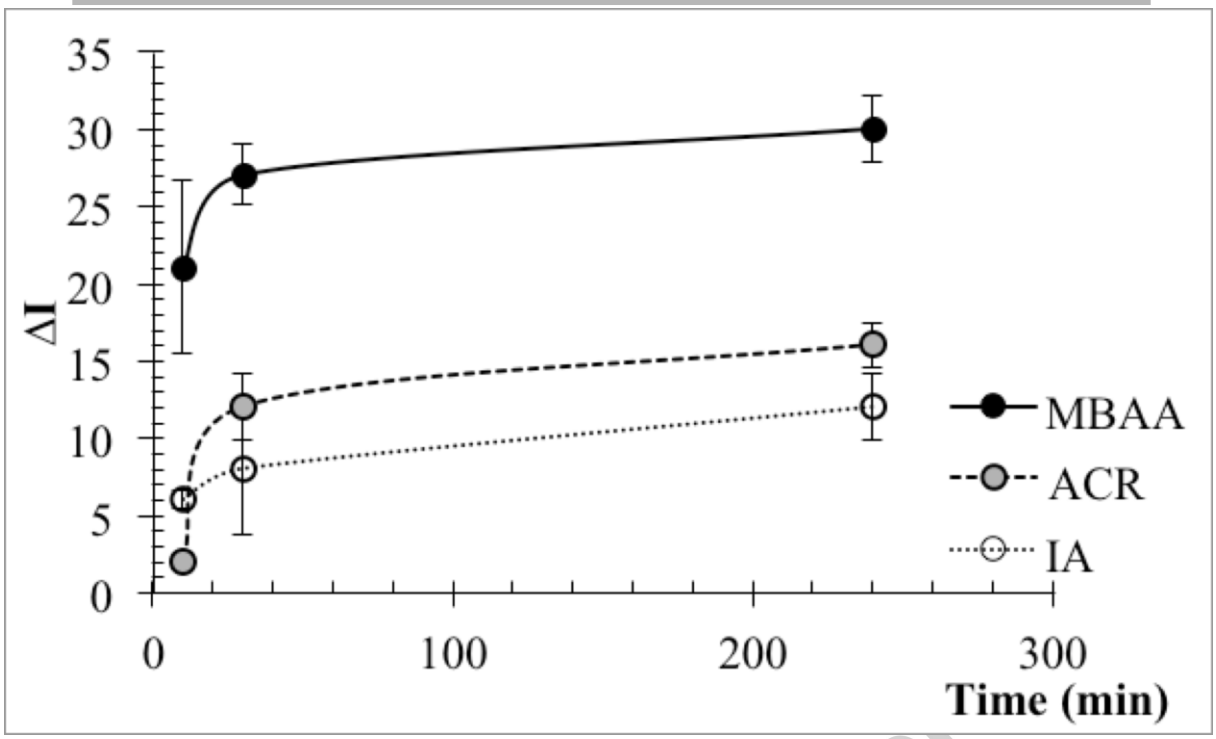
**Highlights**

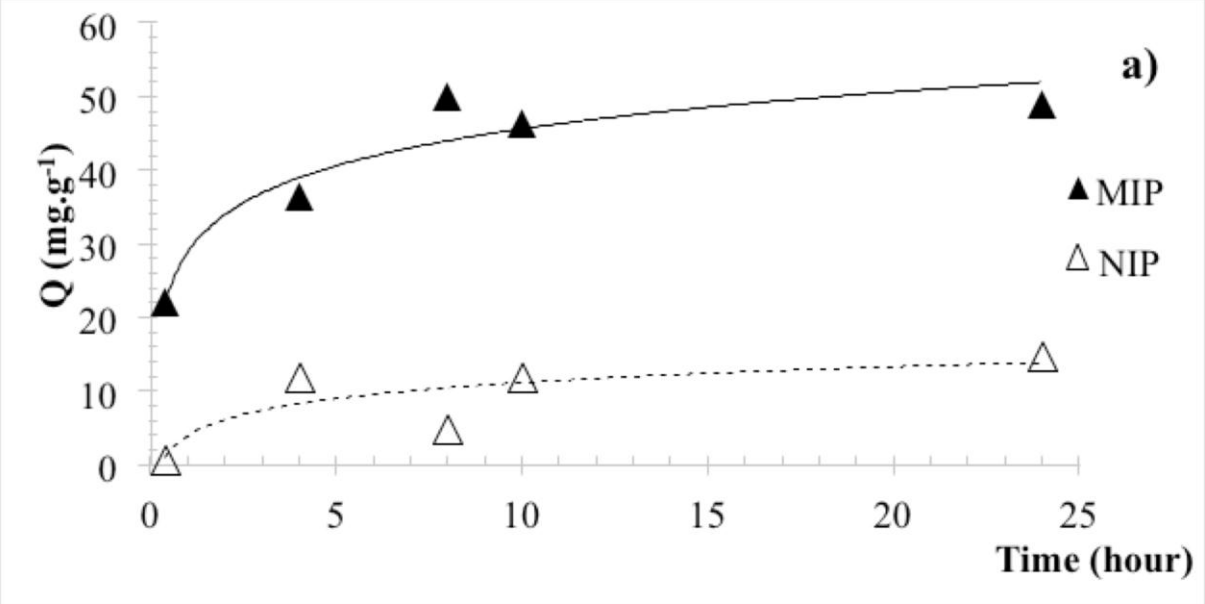
- Molecularly imprinted for phosmet insecticide were synthesized by batch polymerisation
- Appropriate functional monomer and porogenic solvent were selected for the first time using a biosensor
- Molecularly imprinted polymers (MIPs) and non-imprinted polymers (NIPs) were synthesized and adsorption isotherms were studied to describe their interaction with phosmet

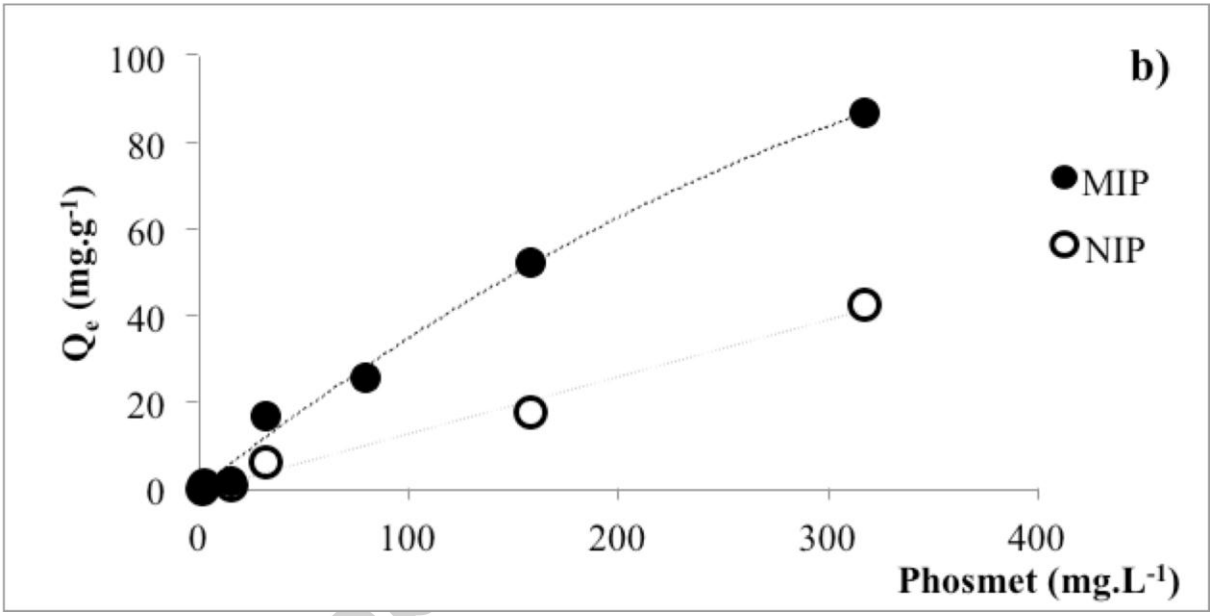









a)
Q (mg.g-1)
60
50
40
30
20
10
0
0
5
10
15
20
25
Time (hour)
MIP
NIP


b)
Qe (mg.g-1)
100
80
60
40
20
0
0
100
200
300
400
Phosmet (mg.L-1)
MIP
NIP