EFFICIENT DETERMINATION AND PESTICIDE CONTROL
BY MEANS OF IMMOBILIZATION OF ACETYLCHOLINESTERASE

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Supports involving tetrazole (o/m/p-F-Tet-1H) were prepared to detect pesticides. This novel tetrazole including fluorine in different positions was attached to nanoparticles (2AEPS-(o/m/p-F-Tet-1H)) by a condensation method. Primarily, the tetrazole derivatives were characterized by ¹H-NMR, ¹³C-NMR and LC-MS. Then, nanoparticles were prepared by a condensation method in non-aqueous medium and characterized by Fourier transform infrared spectroscopy, scanning electron microscopy, gel permeation chromatography. The enzymatic properties of immobilized acetylcholinesterase (AChE) were investigated for the determination of phosmet. This research is the first example.

Keywords: acetylcholinesterase; nanoparticle; hazardous materials; tetrazole; immobilization

1. INTRODUCTION

Tetrazoles are nitrogen-rich stable heterocyclic compounds that are studied extensively due to their wide range of applications [1]. They play important roles in medicinal chemistry [2, 3], and have been important in material science, including as propellants and explosives [4]. Effective methods have been reported in the literature during the past few years for the synthesis of tetrazoles [5]. The use of nanospheres in the synthesis of tetrazoles is one of these methods known to be efficient and economical [6, 7]. Nanospheres have advantages due to their small size and large surface area. These advantages lead to a wide variety of applications as catalyst supports. Nanosphere structures have attracted great attention in preparing advanced materials for biosensing applications [8]. In particular, polymeric nanoparticle materials have attracted significant interest for their potential application in enzyme immobilization [9].

It is known, that enzymes are immobilized by interactions between the support and the enzyme by covalent bonding, hydrogen bonding and
Van der Waals forces [10]. If a polymer-based nanosphere support contains electronegative groups such as oxygen, nitrogen and fluorine this may be useful for increasing the enzyme stability via hydrogen bonding attachment [11]. So, the surface on which the enzyme is immobilized has several vital roles to play, such as retaining the tertiary structure of the enzyme through hydrogen bonding.

Acetylcholinesterase (AChE) is crucial enzyme in the central nervous system of living organisms [12]. The inhibition of AChE activity by organophosphorus (OP) compounds is an irreversible process. In this process, AChE is deactivated. OP compounds, are commonly used as insecticides [13]. Acetylcholinesterase (AChE) is a serine protease enzyme. The inhibition of AChE catalytic activity by OP compounds is caused by the phosphorylation of the serine residue [14].

In this work, new tetrazole derivatives have been prepared as novel supports (Figure 1), designed to take advantage of hydrogen bonding between the enzyme and fluorine, and to improve the recycling stability performance for the determination of hazardous materials. Nanospheres were attached to these derivatives (Figure 2), and acetylcholinesterase then immobilized on the nanoparticles (Figure 3) for practical determination of Phosmet [N-(mercaptomethyl) phthalimide S-(O,O-dimethyl phosphorodithioate)]. Phosmet is a phthalimide-derived organophosphate insecticide used on plants and animals, mainly used on apple trees for the control of codling moth. The experimental results showed that immobilized nanoparticles supports showed excellent results for the determination of pesticides.

![Synthesis rotation of 1-((o-, m-, p-)fluorophenyl)-1H-tetrazole-5-thiol (A) and 2-((o-, m-, p-)fluorophenyl)-1H-tetrazol-5-yl)thio)-5,6-dimethoxy-2,3-dihydro-1H-inden-1-one (B)](image)

**Fig. 1.** Synthesis rotation of 1-((o-, m-, p-)fluorophenyl)-1H-tetrazole-5-thiol (A) and 2-((o-, m-, p-)fluorophenyl)-1H-tetrazol-5-yl)thio)-5,6-dimethoxy-2,3-dihydro-1H-inden-1-one (B)

![Formation of Schiff bases including tetrazole on nanosphere](image)

**Fig. 2.** Formation of Schiff bases including tetrazole on nanosphere
2. EXPERIMENTAL

2.1. Materials and methods

The o-, m-, and p-fluorophenyl isothiocyanates, N-[2-[bis(2-aminoethyl)amino]ethyl]aminomethyl-polystyrene, acetylcholinesterase (Type C3389, from electric eel, 518 units/mg, 10KU), 5,5'-dithiobis(2-nitrobenzoic acid), acetylthiocholine iodide, and N-(mercaptomethyl) phthalimide S-(O,O-dimethyl phosphorodithioate) were purchased from Sigma (St. Louis, MO) and other chemicals were obtained from Sigma-Aldrich. All melting points were determined in sealed capillaries and are reported without correction for tetrazole derivatives. FT-IR/ATR spectra were recorded on a Mattson 1000 spectrometer or a Thermo Nicolet 6700 spectrometer as KBr pellets. 1H-NMR spectra were recorded on a Varian Gemini 300 (300 MHz) NMR spectrometer in dimethyl sulfoxide (DMSO)-d6 and (CDCl3)-d4. 13C-NMR spectra were recorded on a Varian Gemini 300 (75 MHz) NMR spectrometer in dimethyl sulfoxide (DMSO)-d6 and (CDCl3)-d4. Mass spectra measurements were recorded on a Thermo Finnigan Trace DSQ. The GPC measurements were recorded on a Waters 1500 Series Gel permeation chromatography (GPC). Scanning electron microscopy of the Au-Pd-coated compounds was performed by a JEOL JEM 100 CX II scanning electron microscope (JEOL, Peabody, MA) equipped with a Link analytical system. The electron energy was used was 20 keV.

2.2. Preparation of 1-substituted phenyl-1H-tetrazol-5-thiol from organic phenylisothiocyanates

2.3. Preparation of 2-fluorophenyl-1H-tetrazol-5-ylthio)-5,6-dimethoxy-2,3-dihydro-1H-inden-1-one

2.4. Synthesis of nanomaterial (2AEPS-(o/m/p-F-Tet-1H))
2.5. Immobilization of AChE on nanomaterial

2AEPS-(o/m/p-F-Tet-1H)

After dissolving the enzyme in pure water (50 ml, 3.6 × 10^{-4} g 1^{-1}), 2AEPS-(o-F-Tet-1H), 2AEPS-(m-F-Tet-1H) and 2AEPS-(p-F-Tet-1H) polymers (0.5 g) were added to 2 ml of 3.6 × 10^{-4} g 1^{-1} AChE. This solution was diluted to 10 ml and shaken in a water bath at room temperature for 8 h. The immobilized polymers were separated and the free enzyme was removed by washing with phosphate buffer and then stored at +4 °C. The saturation ratio was determined as 94.20, 97.60 and 93.70 % for 2AEPS-(o-F-Tet-1H), 2AEPS-(m-F-Tet-1H) and 2AEPS-(p-F-Tet-1H), respectively, from the absorbance value at 412 nm.

2.6. Assay for enzyme activity measurement

The catalytic activity of acetylcholinesterase (AChE) was determined by the Ellman method. In this method, acetate and thiocohleine forms by hydrolysis of acetylthiocholine (ACh) by AChE. Acetylthiocholine iodide and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) are used as substrates and Ellman's reagent. 2-Nitro-5-thiobenzoate (TNB) forms as a yellow solution in water at neutral and alkaline pH by the reaction of DTNB with acetylthiocholine.

2.7. Study of phosmet insecticide

(N-(mercaptomethyl) phthalimide-S-(O,O-dimethyl phosphorodithioate))

Phosmat insecticide, N-(mercaptomethyl) phthalimide-S-(O,O-dimethyl phosphorodithioate) was dissolved in acetonitrile/H2O (1.37 × 10^{-3} mol/l; 1/4, v/v) in volumes of 10 μl – 50 μl. The absorbance changes at 412 nm were taken into account for studied phosmate solutions.

2.8. Effect of pH and temperature on activity of free and immobilized AChE

The optimum pHs for immobilized and free acetylcholinesterase were determined by measuring the activity of free and immobilized enzymes in buffers of different pH values ranging from 3.0 to 9.5. For this purpose, buffers were prepared: pH 3.0–4.0 (CH3COONa / CH3COOH); pH 5.0 (NaH2PO4 / H3PO4); pH 6 (Na2HPO4 / NaH2PO4); and pH 7.0–9.0 (Na2HPO4 / NaH2PO4).

For the temperature studies, immobilized and free enzymes were incubated in the reaction mixtures at different temperatures ranging from 20 to 90 °C. The activities of free and immobilized enzyme were plotted against temperature.

Optimum pH and temperature for immobilized acetylcholinesterase the following recipe was used: 4 ml studied buffer (pH 3–9) + 20 μl 0.075 M acetylthiocholine + 50 μl 0.01 M DTNB + 20 mg immobilized- acetylcholinesterase. The maximum activity was obtained at pH 8.0 and temperature 40 °C for the free enzyme.

2.9. Effect of substrate

To determine the extent to which immobilization affected the enzyme activity, K_M and V_max were determined at optimum pH and 60 °C. Free and immobilized enzyme were incubated with different substrate concentrations (10 μl – 60 μl 0.075M) in phosphate buffer of pH 8 (4010–3960 μl), and assayed for enzyme activity at 50–70 °C, the recommended temperature for enzyme assays.

2.10. Storage stability and reusability of immobilized enzyme

Storage stability experiments were carried out to determine the stabilities of immobilized enzymes after storage in dry conditions at +4 °C for 5 months. The enzyme activity was measured every 30 days and the observed results compared to the initial activities. To evaluate the reusability, the acetylcholinesterase immobilized polymeric supports were also washed with buffer solution after each run and reintroduced into fresh solution. Reaction cycles under the conditions (pH = 8.0, at room temperature) described above were performed and the enzyme activity measured.

3. RESULTS AND DISCUSSION

3.1. Characterization of 2-fluorophenyl-1H-tetrazol-5-ylthio)-5,6-dimethoxy-2,3-dihydro-1H-inden-1-one

2-(1-(o-fluorophenyl)-1H-tetrazol-5-ylthio)-5,6-dimethoxy-2,3-dihydro-1H-inden-1-one; m.p. 178.3–178.6 °C; yield 74%; IR (KBr): cm^{-1} = 3075 (=C-H), 2883 (-C-H), 1692 (C=O), 1604 (C=C), 1505 (N≡N), 1460 (C≡N), 1016 (C-O-C); 1H NMR (300 MHz, CDCl3); δ = 7.60–7.50 (m, 2H), 7.39–7.31 (m, 2H), 7.20 (s, 1H), 6.89 (s, 1H), 4.71 (dd, 1H, J_{1H,2H} = 7.6 Hz; J_{1H,2H} = 4.2 Hz), 4.00–3.88 (m, 7H), 3.34 (dd, 1H, J_{1H,3H} = 3.7 Hz; J_{1H,3H} = 17.4 Hz) ppm; 13C NMR (75 MHz, CDCl3); δ = 197.7 (C=O), 157.9, 156.7, 154.9, 154.5, 147.7, 133.1, 133.0, 128.1, 127.7, 125.2, 117.5, 107.2

(Ar-C), 150.0 (C=N), 56.4, 56.2 (CH₃), 50.4 (CH), 35.8 (CH₂) ppm; MS: \( m/z = 387.0927 \) [M+1].

2-(1-(4-fluorophenyl)-1H-tetrazol-5-yl)thio)-5,6-dimethoxy-2,3-dihydro-1H-inden-1-one; m.p. 159.7–160.3 °C; yield 69%; IR (KBr): \( cm^{-1} = 3067 \) (＝C＝H), 2955 (＝C＝H), 1708 (C=O), 1594 / 2920 (C=C), 1504 / 2921 (C=C), 1478 (C=C), 131.4, 127.7, 119.4, 117.5, 111.8, 107.2, 104.8 (Ar-C), 150.1 (C=N), 56.4, 56.2 (CH₃) 50.6 (CH), 35.7 (CH₂) ppm; MS: \( m/z = 387.0926 \) [M+1].


2-(1-(4-fluorophenyl)-1H-tetrazol-5-yl)thio)-5,6-dimethoxy-2,3-dihydro-1H-inden-1-one; m.p. 204.5–206 °C; yield 71%; IR (KBr): \( cm^{-1} = 3069 \) (=C＝H), 2940 (＝C＝H), 1696 (C＝O), 1588 (C＝C), 1504 (N＝N), 1470 (C＝O), 1588 (C＝C), 1504 (N＝N), 1470 (C＝O); \(^1\)H NMR (300 MHz, CDCl₃): \( \delta = 7.65–7.59 \) (m, 2H), 7.31–7.23 (m, 2H), 7.22 (s, 1H), 6.90 (s, 1H), 4.74 (dd, 1H, H₁), 3.35 (dd, 1H, H₃, J₃,₁H₁ = 7.6 Hz; J₃,₃H₁ = 4.2 Hz), 4.00–3.90 (m, 7H), 7.34 (dd, 1H, H₁, J₅,₁H₁ = 4.1 Hz; J₅,₅H₁ = 17.4 Hz) ppm; \(^{13}\)C NMR (75 MHz, CDCl₃): \( \delta = 197.6 \) (C＝O), 164.5, 161.1, 156.7, 153.1, 147.8, 131.4, 127.7, 119.4, 117.5, 111.8, 107.2, 104.8 (Ar-C), 150.1 (C＝N), 56.4, 56.2 (CH₃) 50.6 (CH), 35.7 (CH₂) ppm; MS: \( m/z = 387.0926 \) [M+1].

\[ \text{Table 1} \]

| Compound Color | Chemical formula | \( M_w \) (\( M_a \), PDI) | \( \nu_{\text{overton}} \) \( \nu(C=O) \)/\( \nu(CH=N) \)/\( \nu(CH=CH) \) |
|---------------|------------------|------------------|------------------|------------------|------------------|
| 2AEPS-(o-F-Tet-1H) | \([C₂H₅O₂(C₁H₅N₂(C₁H₅C₃O₂S₃N₃F₂))\] | \( (845, 650), 1.30 \) | 1937, 1869, 1801 | 1506, 1462 |
| Yellow, 1582 | \([C₂H₅O₂(C₁H₅N₂(C₁H₅C₃O₂S₃N₃F₂))\] | \( (923, 650), 1.42 \) | 1870, 1801 | 1508, 1465 |
| Orange, 1582 | \([C₂H₅O₂(C₁H₅N₂(C₁H₅C₃O₂S₃N₃F₂))\] | \( (911, 660), 1.38 \) | 1594 / 2920 | 1621 |
| Brown, 1582 | \([C₂H₅O₂(C₁H₅N₂(C₁H₅C₃O₂S₃N₃F₂))\] | \( (911, 660), 1.38 \) | 1594 / 2920 | 1621 |

\( M_a \): Molar weight; \( M_w \): Molar weight distribution; \( *M_a \): was suggested from element analyses

3.3. IR spectra and SEM-EDX of 2AEPS-(o-F-Tet-1H), 2AEPS-(m-F-Tet-1H) and 2AEPS-(p-F-Tet-1H)

The characteristic IR spectra peaks of 2AEPS-(o-F-Tet-1H)-sphere, 2AEPS-(m-F-Tet-1H)-sphere and 2AEPS-(p-F-Tet-1H)-sphere polymers are given in Table 1. As in our previous study, three overtone peaks showed at ca. 19378, 18710 and 1801 cm⁻¹ in the IR spectra of support nanoplatorms attached tetrazole. IR bands ca 2920, 1598, 1508, 1465 cm⁻¹ regions are characteristic of \( \nu(CH) \) aliphatic, \( \nu(C=O) \) aromatic ring, \( \nu(N=N) \) tetrazole ring and \( \nu(C=N) \) tetrazole ring, respectively [19, 20]. The observation of band at ca 1618 cm⁻¹ may be attributed to the \( \nu(CH=CH) \) stretching vibration for 2AEPS-(o-F-Tet-1H), 2AEPS-(m-F-Tet-1H) and 2AEPS-(p-F-Tet-1H). Furthermore, the carbonyl peak (C=O) in tetrazol derivatives disappeared due to formation of the Schiff base. These indicate that the tetrazole derivatives are attached to 2AEPS.
As is known, EDX spectra can provide excellent elemental analysis for all elements in the Periodic Table above beryllium in a modified polymer [21]. Table 2 presents the elemental compositions of synthesized 2AEPS-(o-F-Tet-1H), 2AEPS-(m-F-Tet-1H) and 2AEPS-(p-F-Tet-1H) obtained from EDX analysis. As seen from the SEM image, the spherical structure is unaffected.

| Table 2 |
| SEM image and elemental analysis from EDX for nanosphere-attached tetrazole derivatives |

<table>
<thead>
<tr>
<th>2AEPS-(o-F-Tet-1H)</th>
<th>2AEPS-(p-F-Tet-1H)</th>
<th>2AEPS-(m-F-Tet-1H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>× 1000 mag</td>
<td>× 500 mag</td>
<td>× 200 mag</td>
</tr>
</tbody>
</table>

% C: 72.80; %N: 10.57
%F: 2.38; % S: 3.92
% C: 73.10; %N: 9.97
%F: 2.22; % S: 3.62
% C: 72.56; %N: 10.04
%F: 2.14; % S: 3.80

3.4. Studies of biocatalysis

Firstly, the amount of loaded enzyme per gram of polymer was found according to saturation ratio (s.r.) calculated according to the following formula for all three enzymes:

\[
A_{412} = \varepsilon \times b \times C_{20 \mu l, 0.075 \text{ M}} \\
A_{412(1)} = \varepsilon \times b \times C_{(20 \mu l, 0.075 \text{ M} - \text{immobilized AChE})}
\]

3.5. Influence of pH and temperature on the enzyme activity

pH is one of the important parameters for enzymatic activities in aqueous solution [22]. Free and immobilized supports 2AEPS-(o-F-Tet-1H), 2AEPS-(m-F-Tet-1H) and 2AEPS-(p-F-Tet-1H) showed the same observed maximum activities and are illustrated in Figure 4.

Fig. 4. Effect of pH and temperature on the enzyme activity

Free and immobilized enzyme were incubated (30 min) in the reaction mixtures at different temperatures ranging from 20 to 90 °C. The activities of immobilized enzyme were plotted at optimum temperature. The optimum temperatures for immobilized AChE on 2AEPS-(o-F-Tet-1H), 2AEPS-(m-F-Tet-1H) and 2AEPS-(p-F-Tet-1H) were 50, 60 and 70 °C, respectively (Fig. 4). Free
enzyme had an optimum temperature 40 °C. As shown in Figure 5, the thermal stability of the enzyme increased after immobilization, indicating that the structure of the enzyme is protected at high temperature. The optimum temperature of [2AEPS-(p-F-Tet-1H)]-AChE was higher than that of [2AEPS-(o-F-Tet-1H)]-AChE, [2AEPS-(m-F-Tet-1H)]-AChE. The cause of this may be the impact of hydrogen bonding between the enzyme and fluorine. In [2AEPS-(p-F-Tet-1H)], the hydrogen bonding strength of fluorine in the para position may be more effective.

3.6. Kinetic parameters for free and immobilized AChE

The kinetic parameters of free and immobilized AChE were investigated at different concentrations of substrate (from 186 mM to 1120 mM). The data were plotted as Lineweaver-Burk graphs to calculate V\text{max} and K\text{M} values (Table 3, Fig. 5).

![Kinetic parameters, storage stability and reusability](image)

Fig. 5. Kinetic parameters, storage stability and reusability.

<table>
<thead>
<tr>
<th>Working conditions</th>
<th>Sphere</th>
<th>Supports</th>
</tr>
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<tbody>
<tr>
<td>pH (°C)</td>
<td>Free AChE</td>
<td>[2AEPS-(o-F-Tet-1H)]-AChE</td>
</tr>
<tr>
<td>8.0</td>
<td>50</td>
<td>0.146 / 1.85</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.073 / 4.3</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>0.12 / 1.68</td>
</tr>
</tbody>
</table>

3.7. Storage stability and reusability

As is known, the storage stability of enzymes is one of their most important characteristics; free enzymes lose their activity during storage [24]. Storage stability of the free and immobilized enzymes was determined every month when stored at +4 °C under dry conditions. At the end of the
first month, free enzyme activity decreased (retained 96.55%); however the activity of the immobilized enzyme was preserved for [2AEPS-(o-F-Tet-1H)]-AChE, [2AEPS-(m-F-Tet-1H)]-AChE and [2AEPS-(p-F-Tet-1H)]-AChE, at 99.04, 98.07 and 97.01%, respectively. Storage activity of the [2AEPS-(o-F-Tet-1H)]-AChE was better than [2AEPS-(m-F-Tet-1H)]-AChE and [2AEPS-(p-F-Tet-1H)]-AChE. These results indicate that the immobilized AChE retains its high enzymatic activity, which is very important for the preparation of the proposed enzyme support for industrial application.

As shown in Figure 5, the [2AEPS-(p-F-Tet-1H)], [2AEPS-(o-F-Tet-1H)] and [2AEPS-(m-F-Tet-1H)] were used repeatedly 20 times, and the residual activity was about 13.87, 15.13 and 43.40% of their initial activity, respectively. As shown in Figure 6, the recycling stability performance of [2AEPS-(m-F-Tet-1H)] was better than the other studied supports.

3.8. Evaluation on phosmet insecticide

Changes in absorbance of tetrazole-tagged nanosphere are given in Figure 6; [2AEPS-(o-F-Tet-1H)]-AChE + phosmet insecticide, and [2AEPS-(m-F-Tet-1H)]-AChE + phosmet insecticide, and [2AEPS-(p-F-Tet-1H)]-AChE + phosmet insecticide are shown in order. As it can be seen, there are changes in absorbance due to enzyme–phosmet (inhibitor) complex.

Fig. 6. Spectral change of phosmet insecticide in immobilized sphere + 30 μl (purple line) and immobilized sphere + 50 μl (red line)

Fig. 7. Potential mechanism in between AChE and phosmet
The anionic site and esteric sites of AChE are shown in Figure 7. The inactivity of AChE is dependent on the reaction between the AChE-serine-OH and phosmet. The reaction begins with the nucleophilic attack by the catalytic serine hydroxyl on the carbonyl carbon of the ester bond. We may say that partially electropositive phosphorus is attracted to partially electronegative serine. Thus, one of the sulphur-oxygen bonds is broken; this provides strengthens the phosphorus-enzyme bond. According to Figure 6, the inhibitor effect on 2AEPS-(o-F-Tet-1H)-AChE is greater than others.

4. CONCLUSIONS

Presented for the first time in this study, new ligated tetrazole derivatives on nanospheres have been synthesized for the identification of organophosphates. AChE immobilization has been successfully fabricated for the detection of a pesticide.

The apparent kinetic parameters of the immobilized enzyme and free enzyme were compared, and this showed that the Michaelis constant ($K_M$, $V_{max}$) of the 2AEPS-(o-F-Tet-1H)-AChE was higher than both 2AEPS-(m-F-Tet-1H)-AChE and 2AEPS-(p-F-Tet-1H)-AChE. Probably, stereochemical structure of 2AEPS-(o-F-Tet-1H)-AChE has been protected the three-dimensional structure of the enzyme by means of hydrogen bonds.

Hydrogen bonds are especially important between the hydrogen atoms in the –NH$_2$ / –COOH groups of the enzyme and the fluorine atom in ortho position of the tetrazole molecule, because a five-ring structure may occur with formation of this bond (green shaded region, Fig. 8). So, the three-dimensional structure of the enzyme may be better protected. For this reason, in pesticide determination the 2AEPS-(o-F-Tet-1H)-AChE may more easily interact with phosmet insecticide.

Fig. 8. Hydrogen bonds between enzyme and support material (A), photography related to phosmet insecticide (B)

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