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A NOVEL ACETYLCHOLINESTERASE BIOSENSOR: CORE-SHELL MAGNETIC NANOPARTICLES INCORPORATING A CONJUGATED POLYMER FOR THE DETECTION OF ORGANOPHOSPHORUS PESTICIDES

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KEYWORDS: Conjugated polymer, magnetic nanoparticles, acetylcholinesterase, pesticide biosensor, organophosphorus pesticides.

ABSTRACT

To construct a sensing interface, in the present work, a conjugated polymer and core-shell magnetic nanoparticle containing biosensor was constructed for the pesticide analysis. The monomer; 4,7-di(furan-2-yl)benzo[c][1,2,5]thiadiazole (FBThF) and core-shell magnetic nanoparticles were designed and synthesized for fabrication of the bio-sensing device. The magnetic nanoparticles was first treated with silica and then modified using carboxyl groups which enabled binding of the biomolecules covalently. For the construction of the proposed sensor a two step procedure was performed. First, the poly(FBThF) was electrochemically generated on the electrode surface. Then, carboxyl group modified magnetic nanoparticles (f-MNPs) and acetylcholinesterase (AChE), the model enzyme, were co-immobilized on the polymer coated surface. Thereby, a robust and novel surface, "conjugated polymer bearing magnetic nanoparticles with pendant carboxyl groups", was constructed which was characterized using FT-IR spectrometer, cyclic voltammetry (CV), scanning electron microscopy (SEM) and contact angle measurements. This novel architecture was then applied as an immobilization platform to detect pesticides. To the best our knowledge, a sensor design which combines both conjugated polymer and magnetic nanoparticles was attempted for the first time and this approach was resulted in improved biosensor characteristics. Hence, this approach opens a new perspective in the field of enzyme immobilization and sensing applications. Paraoxon and trichlorfon were selected as the model toxicants. To obtain best biosensor performance, optimization studies were performed. Under optimized conditions, the biosensor in concern revealed a rapid response (5 s), a low detection limit (6.66×10^{-3} mM) and high sensitivity ($45.01 \mu A mM^{-1} cm^{-2}$). The K_m^{app} value of poly(FBThF)/f-MNPs/AChE were determined as 0.73

mM. Furthermore, there was no considerable activity loss for ten days for poly(FBThF)/f-MNPs/AChE biofilm.

1. INTRODUCTION

Organophosphorus pesticides (OPs) and their derivatives are known as toxic agricultural wastes which are still widely used in agriculture. The majority of pesticides are potentially toxic for human health since the pesticide residue in the environment can cause high acute toxicity or long-term damage to human health, such as bioaccumulation. Although their use helps to increase the quantity of products, pesticides may enter human diet which might result in a potentially lethal cholinergic poisoning since they alter the catalytic activity of AChE by producing a stable complex in the main site of AChE.[1,2] Serine residue is blocked with inhibition causing a drastic accumulation of ACh which hinders neurotransmission eventually leading to death. To protect humans from the risk related to pesticides in food and environmental water, designing a basic, rapid and a cheap analysis method for the detection of pesticides is of great importance.

Thus far, traditional techniques for the detection of pesticides are based on analysis methods such as gas and/or liquid chromatography. In spite of their high sensitivity and reliability, these methods have some disadvantages such as need for high cost experimental instrumentation and trained personnel, long analysis times and complex sample pretreatment.[3] On the other hand constructing enzyme-based electrochemical biosensors have aroused great interest among academia due to their easy measurement procedure, quick response, potential for miniaturization, excellent sensitivity and selectivity toward analytes. Biosensors are commonly utilized in many areas of clinical analysis and food industry.[4,5] A biosensor device broadly classified on the basis of a sensitive bio-recognition material together with a transducer element that converts the

biological response into electrical signal. OP monitoring is one of the hot topics in biosensors since accurate determination of OPs is a considerably important factor for human health protection. Therefore, in the last decades, a number of efforts have been devoted to the biosensor construction for biochemical analyses due to their high sensitivity, stability and reproducibility.[6,7] Amperometric acetylcholinesterase biosensors have been proposed for monitoring water pollution or other potential risks to human health. Various type of the amperometric AChE based biosensors have been developed.[2,8,9] ,

AChE is an enzyme that utilized in biosensors for determining organophosphate pesticides. The inhibition mechanism involves high affinity of OPs toward the AChE enzyme where the hydroxyl of the serine within the active side is phosphorylated.[10,11] The enzyme mechanism is based on measuring the oxidative current of acetylthiocholine upon applied potential.

Conjugated polymers (CPs) with an extended π-electron system have been widely used as a component in several applications including field effect transistors, organic photovoltaic cells, electrochromic devices and biosensors.[12,13,14] CPs are an important class of material for biomolecule based technology, since they provide homogenous and manageable film character, stability and biocompatibility, reproducibility and ease of production. The use of CPs in biosensor construction enables creative opportunities for the fabrication of biosensors. Especially electrochemical methods for film formation lead to precise control of polymer formation on the transducer surface. Transducers fabricated via these methods offer extensive stability for the enzyme immobilization.[15] Due to these improve properties, researchers have been developing effective bio-sensing platform using CPs. Guler and her colleagues developed a biosensor and showed that use of the amino functionalized CPs provided targeted immobilization of bio-components and also improved the stability of bio-components due to the covalent-type

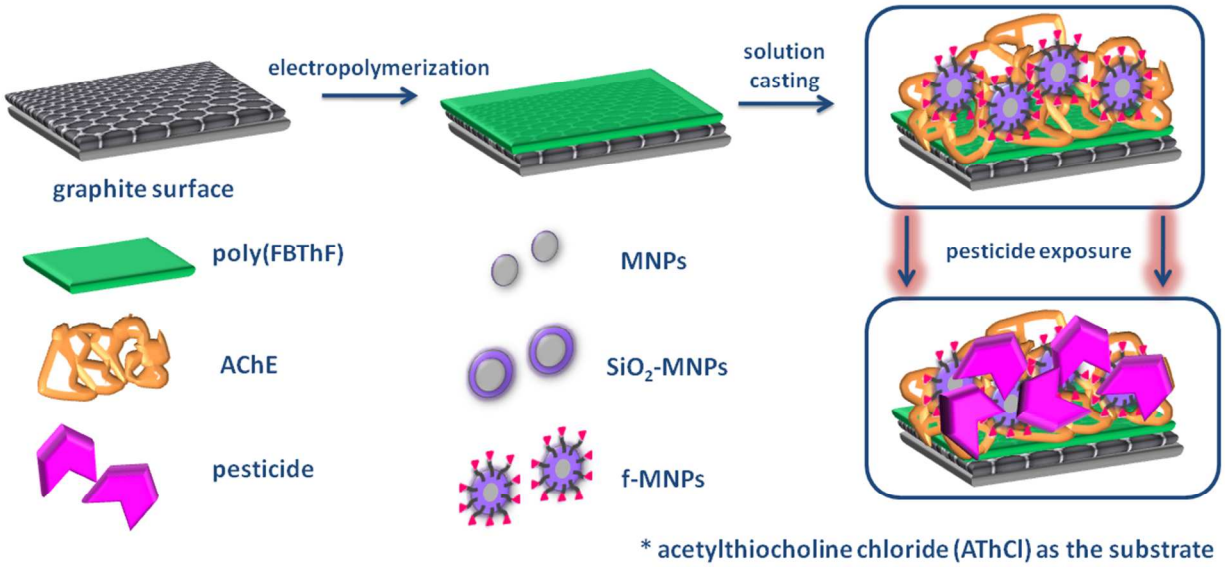
immobilization.[16] In another study, Guler et. al. prepared a novel macro-monomer, (T-g–PPhe) and used its polymer as immobilization matrix for a biosensor. They reported polymeric surface improves the biosensor characteristics.[17] Wan et. al. designed an amperometric glucose biosensor using a combination of chitosan carbon nanotubes (CS-CNTs) composites with polyaniline (PANI)-modified gold electrode. Presence of polymer and CS-CNTs offered a biocompatible environment and enhanced the activity of the enzyme.[18] Moreover; our group also fabricated various CP based electrochemical biosensors for the determination of analytes such as cholesterol, glucose, alcohol and pesticides.[19-22]

The magnetic nanoparticles have been used in many areas of research such as magnetic resonance imaging, drug targeting, gene therapy and biosensor technologies.[23,24]. Magnetite (Fe_3O_4) and maghemite (γ -Fe_2O_3) are popular magnetic nanoparticles for biological studies.[25] When the size of iron oxide nanoparticles is less than 20 nm, they are called super paramagnetic nanoparticles. These types of iron oxide nanoparticles are favorable due to their high field irreversibility and high saturation field.[26] Moreover, iron oxide nanoparticles have high chemical stability and low toxicity in contrast to pure iron nanoparticles.[27] Stability, high hydrophilicity and biocompatibility are required properties for nanoparticles in biological applications. For this purpose, surface of magnetic nanoparticle were coated with various organic or inorganic materials such as poly (lactic-co-glycolic acid), polyethylene glycol (PEG), dextran and silica.[28,29] Silica coating prevents agglomeration of magnetic nanoparticles and increase bio-compability. Furthermore, it provides a suitable platform for further modification of the surface by various functional groups.[30] Since magnetic nanoparticles based biosensors offer a sensitive tool and allow fast detection of analytes, several biomolecules such as glucose oxidase and AChE enzymes have been immobilized on the electrode surface coated with iron oxide

nanoparticles.[31,32] In this study, core-shell magnetic nanoparticles were synthesized and then modified with silica and carboxyl group, respectively. The inner shell of silica with outer skeleton of carboxyl groups of magnetic nanoparticle core not only stabilizes the nanoparticles in solution but also provides sites of covalent attachment for biomolecules. In order to keep a favorable enzyme orientation during the biosensor construction, the adopted immobilization method is the key consideration. An appropriate immobilization matrix should be biocompatible, non-toxic to bio-recognition element, durable and should possess appropriate functional groups for immobilization. In addition, after the immobilization procedure, the enzyme molecules should strongly attach onto the electrode surface, maintaining its native catalytic activity and random orientation. These factors result in suitable operational conditions while keeping enzyme activity intact. Among the existing immobilization techniques, covalent immobilization is the most preferred method for fabrication of a stable and cost-effective immobilization platform.

The motivation of this study was to design an effective bio-recognition interface for the pesticide detection. For this reason, 4,7-di(furan-2-yl)benzo[c][1,2,5]thiadiazole (FBThF) was electrochemically polymerized onto the graphite electrode. This polymeric surface was then modified with f-MNPs and the model enzyme acetylcholinesterase (AChE). Here, we anticipated that the combination of poly(FBThF) and f-MNPs would lead to the formation of a suitable matrix compared to those constructed using either of poly(FBThF) or magnetic nanoparticles. Through functional carboxyl groups of the magnetic nanoparticles, amide bond formation between enzyme molecules and polymer was achieved. Generated immobilization matrix has the advantage of having modified surfaces which contribute fast and selective determination of the pesticides. There are no reports offering this type of biosensor containing combination of polymer and magnetic nanoparticles for detection of paraoxon and/or trichlorfon. Schematic

representation for the preparation of poly(FBThF)/f-MNPs/AChE/graphite electrode biosensor is shown in Scheme 1.



Scheme 1. Schematic representation of the proposed biosensor.

2. EXPERIMENTAL SECTION

Acetylcholinesterase (AChE) (E.C.3.1.1.7) (518 U/mg protein) from *Electrophorus electricus* (electric eel), acetylthiocholine chloride, paraoxon, trichlorfon and chemicals used in electropolymerization were supplied by Sigma-Aldrich. N-Hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) were obtained from Fluka (Buchs, Switzerland) and Sigma, respectively. Dichloromethane (DCM), acetonitrile (ACN), ethanol and glutaric anhydride ($C_4H_4O_3$) were purchased from Merck (Darmstadt, Germany). Iron (III) chloride reagent grade, >97%, iron (II) chloride tetrahydrate, 99%, sodium hydroxide pellets, 3-aminopropyl trietoxysilane APTES, ($C_9H_{23}NO_3Si$), tetraethyl orthosilicate, TEOS, ($C_8H_{20}O_4Si$), 98%, and N,N-Dimethylformide, DMF, ≥99.0 % and the chemicals used in the

synthesis of tributyl(furan-2-yl)stannane, 2,1,3-benzothiadiazole were purchased from Sigma Aldrich. Ammonium hydroxide 26% puriss, was obtained from Riedel-De HAEN.

2.1. Apparatus

All electrochemical experiments were done using potentiostat EmStat (PalmSens, Houten, The Netherlands, www.palmsens.com) in a cell consisting of a graphite electrode (Ringsdorff Werke GmbH, 3.05 mm diameter and 13% porosity) as the working electrode. The auxillary electrode was Pt wire where the reference was a Ag wire. Scanning electron microscope (SEM) (JEOL JSM-6400 model) was used for investigating biosensor architecture. Also, FEI Tecnai G2 Spirit BioTwin transmission electron microscope (TEM) was used for magnetic nanoparticle images. Cyclic voltammetry studies were performed with a GAMRY Reference 600 (GAMRY Instruments Inc., Pennsylvania, USA). Structural analysis of the monomer was performed by nuclear magnetic resonance (NMR) on a Bruker Spectrospin Avance DPX-400 Spectrometer with trimethylsilane (TMS) as the internal reference. The sessile drop method with a CAM100 KSV instrument (KSV, Finland) was used to measure the contact angle of 2.0 μL water on the surfaces. A CCD camera was utilized to observe the differences in contact angle via recording the drop profile. FTIR Spectroscopy (Alpha, Bruker) was used as the proof of nanoparticles in the range of 375–4000 cm^{-1}.

2.2. Synthesis of 4,7-di(furan-2-yl)benzo[c][1,2,5]thiadiazole (FBThF)

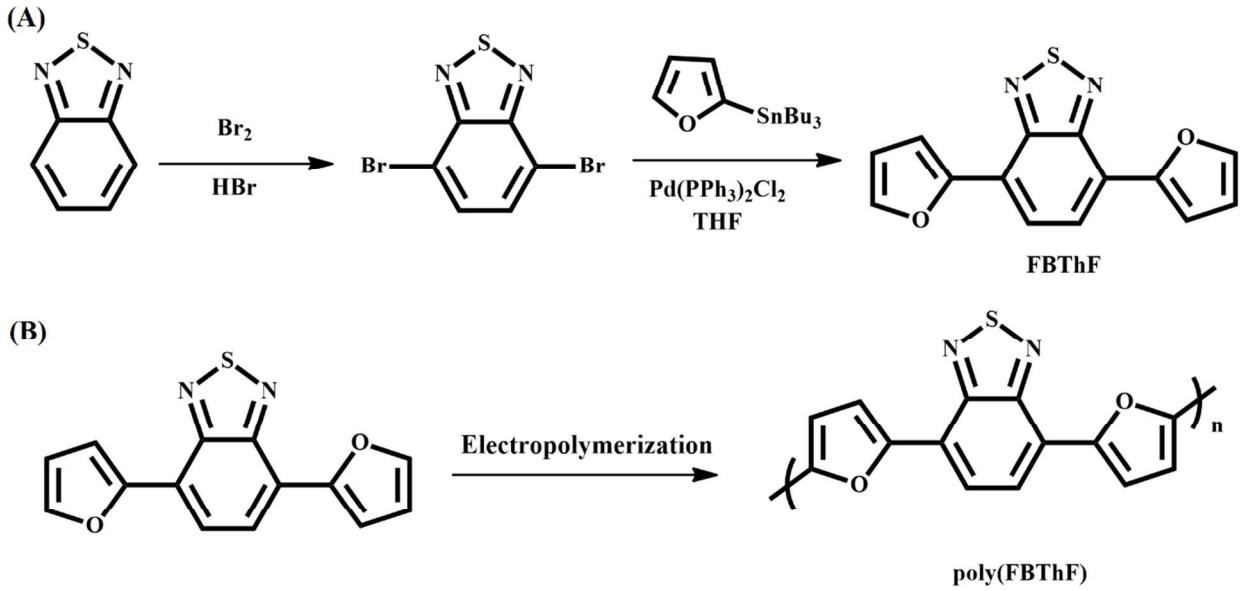
Method of Yuan et. al. was used with small modifications.[33] 4,7-Dibromobenzo[c][1,2,5]thiadiazole[34] (300 mg, 1.02 mmol) and tributyl(furan-2-yl)stannane (1.09 g, 3.06 mmol) were dissolved in dry THF (25 mL). The reaction mixture was heated to

reflux under argon atmosphere for 1 hour and then $PdCl_2(PPh_3)_2$ (71.6 mg, 0.102 mmol) was added. The mixture was refluxed under inert atmosphere for 2 days. The solvent was evaporated and the crude product was purified by column chromatography (hexane/chloroform (1:3)) to afford an orange solid. Yield: 88%. The synthetic pathway of the FBThF was shown in Scheme 2.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.04 (s, 2H), 7.69 (d, J = 3.4 Hz, 2H), 7.59 (d, 2H, J = 2.7 Hz), 6.63 (dd, 2H, J_1 = 3.4 Hz, J_2 = 1.8 Hz)

^{13}C-NMR (100 MHz, $CDCl_3$): δ 151.3, 150.2, 142.8, 123.5, 112.8, 112.5, 112.2

HRMS (ESI) for $C_{14}H_8N_2O_2S$ calculated 269.0385, found 269.0387.



Scheme 2. (A) Synthetic pathway and (B) electropolymerization of FBThF.

2.3. Synthesis of Fe_3O_4@SiO_2@COOH

2.3.1. Synthesis Fe_3O_4 nanoparticles (MNPs)

Coprecipitation method was used for the preparation Fe_3O_4 NPs (MNPs).[35] 50 µL HCl, 0.25 g of $FeCl_3$ and 0.65 g of $FeCl_2$ were dissolved in 3.25 mL deoxygenated deionized water where molar ratio of Fe(II)/Fe(III) was adjusted to 0.5. This solution was added drop wise to 31.25 mL, 1.5 M NaOH solution under stirring. System was purged with nitrogen gas to prevent oxidation. After 1 h stirring, black precipitate was obtained and washed 3 times with water/ethanol mixture. Finally, MNPs were dispersed in 50 mL water.

2.3.2. Preparation Fe_3O_4 @SiO_2 nanoparticles (SiO_2-MNPs)

Silica coating of MNPs was done according to Stöber Method.[36] MNPs were first dispersed in water. 14 mL of this dispersion was diluted to 500 mL with O_2 free deionized water. 2.5 mL of 1mM (3-aminopropyl) trietoxysilane (APTES) were added to this solution and stirred for 15 min. Particles was collected by magnet and transferred to 500 mL deionized water/ethanol (1:4) solution. 300 µL tetraethyl orthosilicate and 2 mL ammonia solution were added and the mixture was stirred for 12 h. Under the influence of magnetic field, SiO_2-MNPs were collected and washed with deionized water two times and redispersed in 50 mL ethanol.

2.3.3. Modification of Fe_3O_4 @SiO_2 nanoparticles by carboxyl group (f-MNPs)

Surface modification of SiO_2-MNPs with amine groups was done according to the procedure given by Ma et. al.[37] 25 mL SiO_2-MNPs were diluted to 60 mL with ethanol. Following the addition of 400 µL deionized water and 300 µL APTES, the mixture was stirred for 12 h. Under the influence of magnetic field, amine modified SiO_2-MNPs were collected and washed with ethanol five times and dispersed in 25 mL N,N-dimethylformamide (DMF).

Amine modified SiO_2-MNPs were further functionalized with carboxyl group by a ring opening linker elongation reaction which occurs in between amine group and glutaric anhydride. For this purpose, 10 mL amine modified SiO_2-MNPs solution in DMF were added to 10 mL DMF solution containing 0.1 g glutaric anhydride and stirred for 24 hours. Under the influence of magnetic field, carboxyl group modified SiO_2-MNPs (f-MNPs) were washed with DMF two times and redispersed in 10 mL deionized water.[38]

2.4. Biosensor Preparation

Before each experiment, graphite rods were polished carefully on an emery paper and cleaned with distilled water. After cleaning procedure, poly(FBThF) was coated on the electrode surface via 55 voltammetry cycles (conditions are given in the figure caption Fig. 1). Then, polymer coated surface was washed thoroughly with distilled water to remove impurities. To prepare the poly(FBThF)/f-MNPs/AChE/graphite electrode biosensor, 10 μL of the solution (f-MNPs/AChE/EDC/NHS) were casted on a freshly prepared poly(FBThF) coated surface and allowed to dry for 3 h under ambient conditions. Then, the electrode was stored overnight. Before use, the electrode surface was washed with distilled water to remove the unbound particles as well as the unbound enzyme. The biosensor construction method involves the making of amide bonds between the carboxyl group modified MNPs and biomolecules. In order to increase the stability and fabricate long-life biosensors, covalent immobilization technique was chosen. The electrode was kept at 4 ºC when not in use.

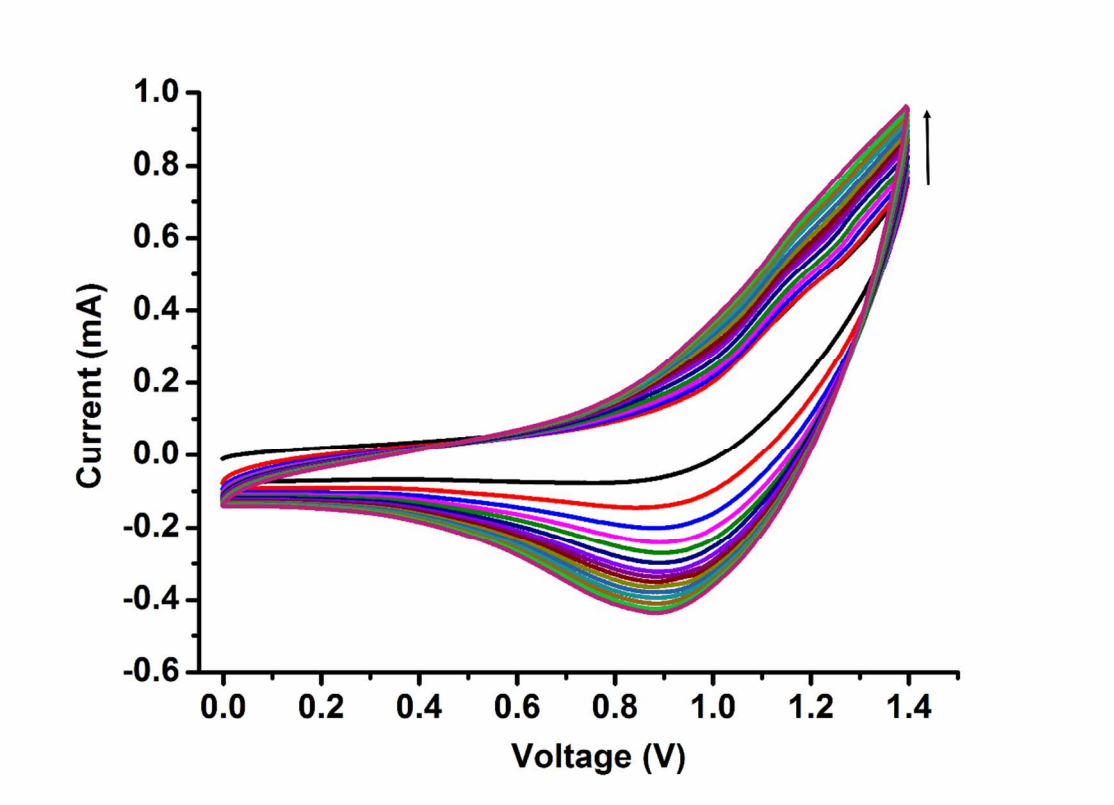


Figure 1. Repeated potential scan electropolymerization of FBThF at 100 mV/s in 0.1 M $TBAPF_6$/DCM/ACN solution by sweeping the potential between 0.0 and 1.4 V on graphite electrode (15 cycles were shown).

2.5. Measurement Procedure

For amperometric biosensor measurements, 10 mL 50 mM phosphate buffer solution (PBS) (pH 7.5) were placed in cell with mild stirring. After a steady state in current was reached, a certain amount of acetylthiocholine chloride (AThCl) was added into the reaction medium as the substrate. The response of the biosensor was measured after a steady state current was achieved. After each measurement, the electrode was washed with distilled water and the buffer was replaced with fresh solution. During the experiments substrate solution was prepared freshly.

A two-step procedure was carried out for inhibition tests. Prior to pesticide incubation, the initial response of the biosensor for substrate was determined. It was then washed and dipped into the pesticide solution for 10 min of incubation. After that, the biosensor was washed with distilled water and PBS. The inhibition rate of pesticides was obtained as the relative decrease in the response:[39]

$I = I_0 – I_1/ I_0 \times 100$

where I_0 is the biosensor response before incubation where I_1 refers to the one after incubation..

Tap water samples from Ankara, Turkey were spiked with the solution of the corresponding pesticides (paraoxon and trichlorfon) to obtain concentrations of 0.05 and 0.814 μg/L. The concentrations were determined with the biosensor following two-step inhibition procedure as described above.

3. RESULTS AND DISCUSSION

3.1. Characterization of Magnetic Core–Shell Nanoparticles

The procedure reported by Kang et al. was used due to the formation of smaller spherical shaped MNPs.[35] As can be seen from the SEM image, Fig. 2, the mean size of the MNPs is about 12 nm. Additionally they have very narrow size distribution between 8 and 12 nm. MNPs were coated with silica shell by using Stöber method. As depicted in Fig. 2, they have homogeneous shape and size distribution. The average shell thickness of SiO_2-MNPs was calculated as 2 nm (Fig S1).

FTIR spectra of MNPs, SiO_2-MNPs and f-MNPs were shown in figures S2A-D. Peak positions were marked with dotted lines and numbers (1-6) were given to the characteristic peaks. Typical

peak of Fe-O was observed at 580 cm $^{-1}$ (1) for all spectra.[40] Asymmetric stretching of Si-O vibration peak at 1079 cm^{-1} (2) was observed in spectra B and C, indicating the presence of silica layer on the surface of MNPs. Characteristic amide I band at 1640 cm^{-1} (5) and amide II band at 1558 cm^{-1} (4) were depicted in spectrum D. The presence of amide band was an evidence for the existence of -NHCO- group that was the product of the reaction between $–NH_2$ and glutaric anhydride. 1737 cm^{-1} (6) belonged to carbonyl group stretching and the peak at 1421 cm^{-1} (3) was attributed to COH in plane bending of carboxylic acid group.[41]

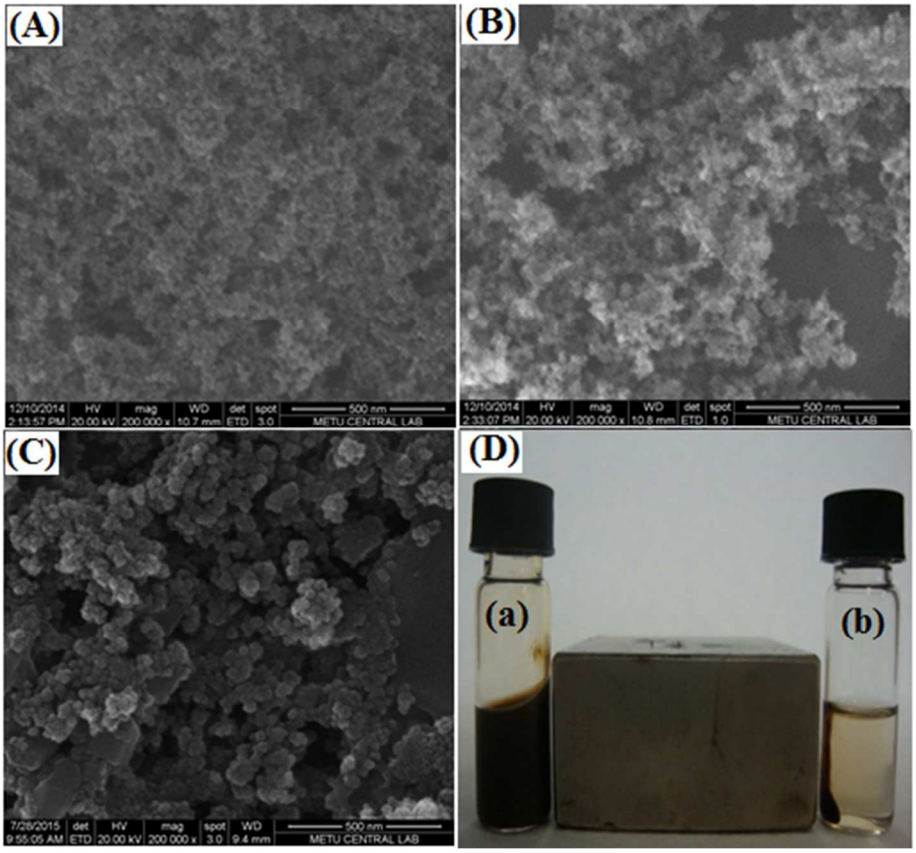


Figure 2. SEM image of (A) MNPs with dimensions in the range of 8-12 nm, (B) SiO_2-MNPs with dimensions in the range of 12-17 nm, (C) f-MNPs with dimensions in the range of 35-45 nm, and (D) Behavior of MNPs under an external magnetic field a) original solution b) the same solution after 30 min magnetic field application.

3.2. Optimization studies of the biosensor

The effect of several parameters such as, pH, thickness of the polymer film, amount of enzyme and f-MNPs were studied to obtain maximum amperometric response during the measurements. First, the effect of polymer thickness on the sensitivity of the AChE biosensor was optimized. Polymer thickness on the electrode surface was determined using cyclic voltammetry. Towards this aim, different matrices with 10, 30, 45, 55, 65 and 70 scans were prepared. As seen in Fig. S3A, the best results were obtained with 55 cycle deposition corresponding to 12.9 μm thickness. Very thick film may cause a diffusion problem between the polymer coated transducer and the biomolecule resulting in a lower charge transfer rate. On the contrary, 3D structures of the biomolecule on the very thin film may cause a low amperometric response.

The pH of the buffer is essential to evaluate the sensitivity of the biosensors since the activity of biomolecules and the stability of MNPs are dependent on the pH of the medium. Changes in pH may not only affect the activity of the biomolecules but may also affect the interaction between the substrate and the active site of biomolecules. Harsh environments would decrease the activity and 3D structure of the enzyme, leading to a decrease in electrochemical response. The pH was varied from 6.5 to 8.0. The amperometric responses were tested in several pH values (Fig. S3B). The maximum response was observed at pH 7.5.

Enzymes should be sufficiently oriented on the electrode surface so as to obtain a broad linear response range. Biosensors were prepared with different AChE amounts between 0.5 U and 1.5 U where other parameters were kept constant. The highest amperometric response was obtained with the biosensor prepared using 1U AChE. A larger amount of enzyme leads to less bonding with the transducer and can easily leach out from the surface. On the other hand, lower amounts

of enzyme molecule may cause inadequate enzymatic reaction resulting in unstable current response. 1U enzyme was used in all further experiments (Fig. S3C).

Furthermore, to get the best combination for a high biosensor performance, f-MNPs content was tested. Different concentrations of f-MNPs were prepared. As shown in Fig. S3D, when f-MNPs amount was lower than the optimum amount of f-MNPs, enzyme molecule could not be fixed onto the polymer coated surface which results in leaching of enzyme from the electrode surface. On the other hand, higher amount of f-MNPs exhibit significantly reduced amperometric responses. One drawback of existing higher amount of f-MNPs is that the enzyme activity decreases significantly due to the changes in enzyme 3D structure or limited access of substrate to the active site of the enzyme. All these factors cause enzyme denaturation and limit the practical application of the final biosensor.

3.3. Effect of f-MNPs and poly(FBThF) on Biosensor Performance

Next effect of combined use of nanoparticles (f-MNPs) and conducting polymer (poly(FBThF) on performance of acetylcholinesterase biosensor was evaluated. For this purpose, several electrodes were prepared and their amperometric results were compared. As seen in Fig. 3, amperometric signals of pristine poly(FBThF) on the electrode surface, was lower compared to the to the electrode where polymer was modified with f-MNPs. Pristine conjugated polymer was not able to bind the enzyme molecules on the electrode surface completely. Although conjugated polymers serve as a valuable host for biomolecule binding which leads to long lasting sensors, it may not be highly effective to obtain proper orientation and binding of enzymes on the electrode.[42] On the other hand, modification of the polymeric surface with f-MNPs led to an increase in the biosensor response as well as served as an appropriate platform for enzyme

deposition. MNPs have gained significant attention since they provide rapid detection of analytes as well as improving the biosensor performance. It was observed that there was an issue in fixing the enzyme molecules for the biosensor prepared with just f-MNPs due to the incompatibility of the enzyme with the f-MNPs modified surface. Moreover, during the amperometric measurements, unstable amperometric signals were recorded although f-MNPs have carboxyl groups which are open to amide bonding. It was difficult to obtain consecutive reliable measurements. In addition, it was noted that enzyme molecules leached from the electrode surface after a few measurement. Because of these abnormal results, we did not report any amperometric results for pristine f-MNPs biosensor. However, in the absence of f-MNPs, binding of AChE to polymeric surface was insufficient and resulted in low activity. Through the use of f-MNPs, the sensitivity of the biosensor was increased by as much as 3-fold. Hence, to obtain best biosensor performance, f-MNPs and polymer surface were both essential for immobilization of AChE enzyme onto the electrode surface.

In the present study, f-MNPs were modified with carboxyl group to enhance biosensor performance. Covalent bonds were used to combine carboxyl groups of f-MNPs and amine groups of the enzyme molecule. In addition to this, strong π–π stacking enhances the enzyme immobilization and this results in stabilized tertiary structure of the enzyme. Hence, all these binary interactions improved the immobilization of the biomolecule without losing its biocatalytic activity. Combining such unique properties leads to significant improvements in biosensor performance. The studies proved that novel bio-surface shows better enzyme stability with higher analytical parameters compare to the ones where individual components were utilized.

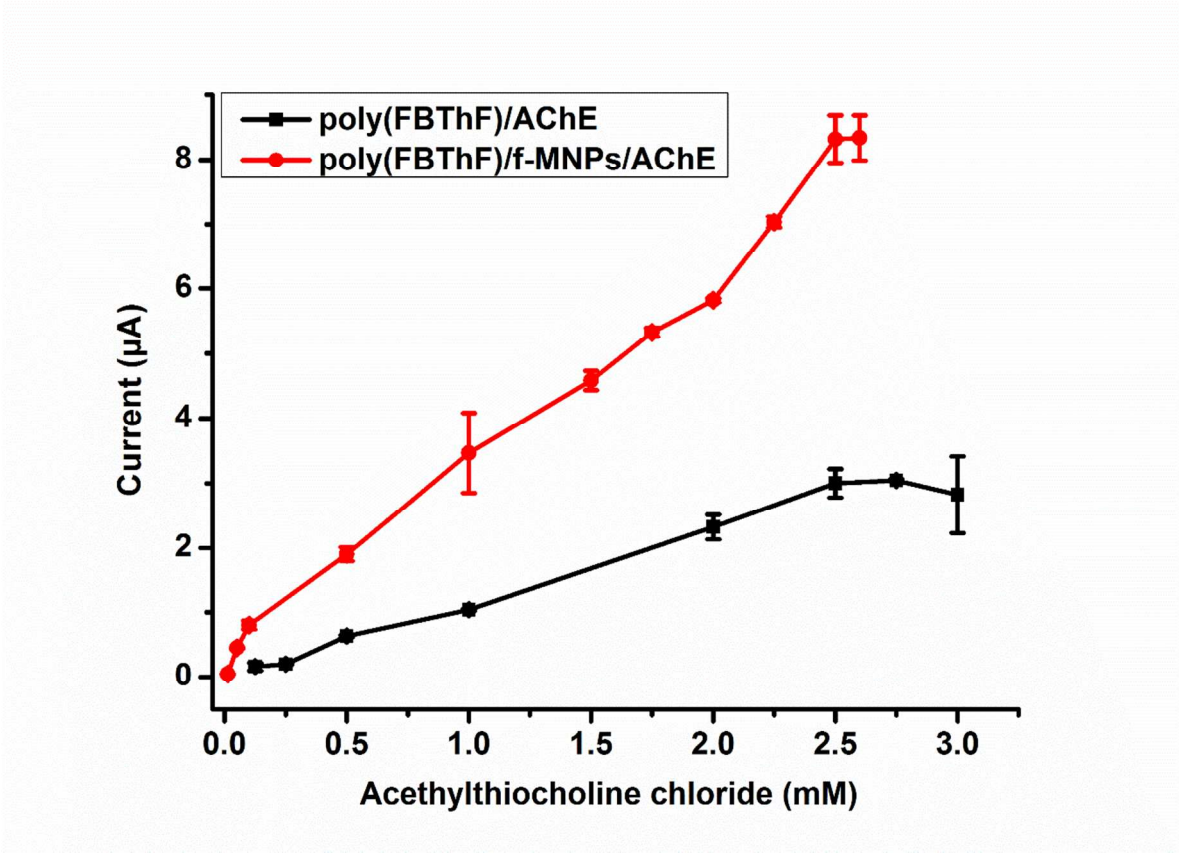


Figure 3. Effect of f-MNPs on poly(FBThF)/f-MNPs/AChE biosensor (in 50 mM PBS, pH 7.5). Error bars show the standard deviation (SD) of three measurements.

3.4. Surface Characterizations of the Electrodes

Cyclic voltammetry studies in 5 mM $Fe(CN)_6^{3-/4-}$ containing 0.1 M KCl (20 mVs^{-1} as scan rate) varified the surface modification of modified electrodes (Fig. 4). An oxidation peak at ca. 0.2 V with a current of 39 μA was monitored for poly(FBThF) coated graphite electrode. Incorporation of f-MNPs onto the conducting polymer matrix resulted in an oxidation peak with a higher current (58 μA). This phenomenon is due to the presence of f-MNPs which increases the electron mobility. This is in return cause a promoted electron transfer. High specific surface area and inherent high electrical conductivity of the nanoparticles enhance the electron transfer. After the immobilization of AChE onto the electrode the oxidation peak current decreased to 45 μA

confirming the satisfactory attachment of the biomolecule. This was due to the insulator nature of the biological molecules hindering the transfer of electrons to electrode surface.

The average value of the electroactive surface was calculated according to a previous report[43] .

The electroactive surface area for poly(FBThF), poly(FBThF)/f-MNPs and poly(FBThF)/f-MNPs/AChE modified electrodes were 7.7 mm^2, 11.5 mm^2 and 9.8 mm^2, respectively. Accordingly, poly(FBThF)/f-MNPs electrode exhibited the highest oxidation peak current resulted in the highest electroactive surface area.

The surface morphology of poly(FBThF) and poly(FBThF)/f-MNPs/AChE were characterized using scanning electron microscope (SEM). The SEM image of poly(FBThF) coated graphite electrode (Fig. 5A) displayed typical cauliflower like structure of conducting polymers. The uniform and homogeneous coating exhibited a three-dimensional structure with a large surface area which is a favorable environment for high loadings of enzyme. After AChE was immobilized onto f-MNPs distributed on the polymer, the SEM image obtained for the poly(FBThF)/f-MNPs/AChE electrode and the images showed globular shape on the surface (Fig. 5B). The results suggested that f-MNPs/AChE immobilization was successfully achieved by the cross linker.

Moreover, the changes in surface hydrophilicility before and after enzyme immobilization can be assessed qualitatively by measuring the contact angles of water. In this technique, ability of water to spread over the surface was studied by averaging the left and right angles of drops with the same volume. For the electrode coated with poly(FBThF), a contact angle of 84.12 ± 1.47º was found owing to highly hydrophobic character of the conducting polymer film. Co-immobilization of f-MNPs and AChE led to a decrease in the contact angle (64.83±0.73º) revealing successful immobilization on the polymer modified transducer.

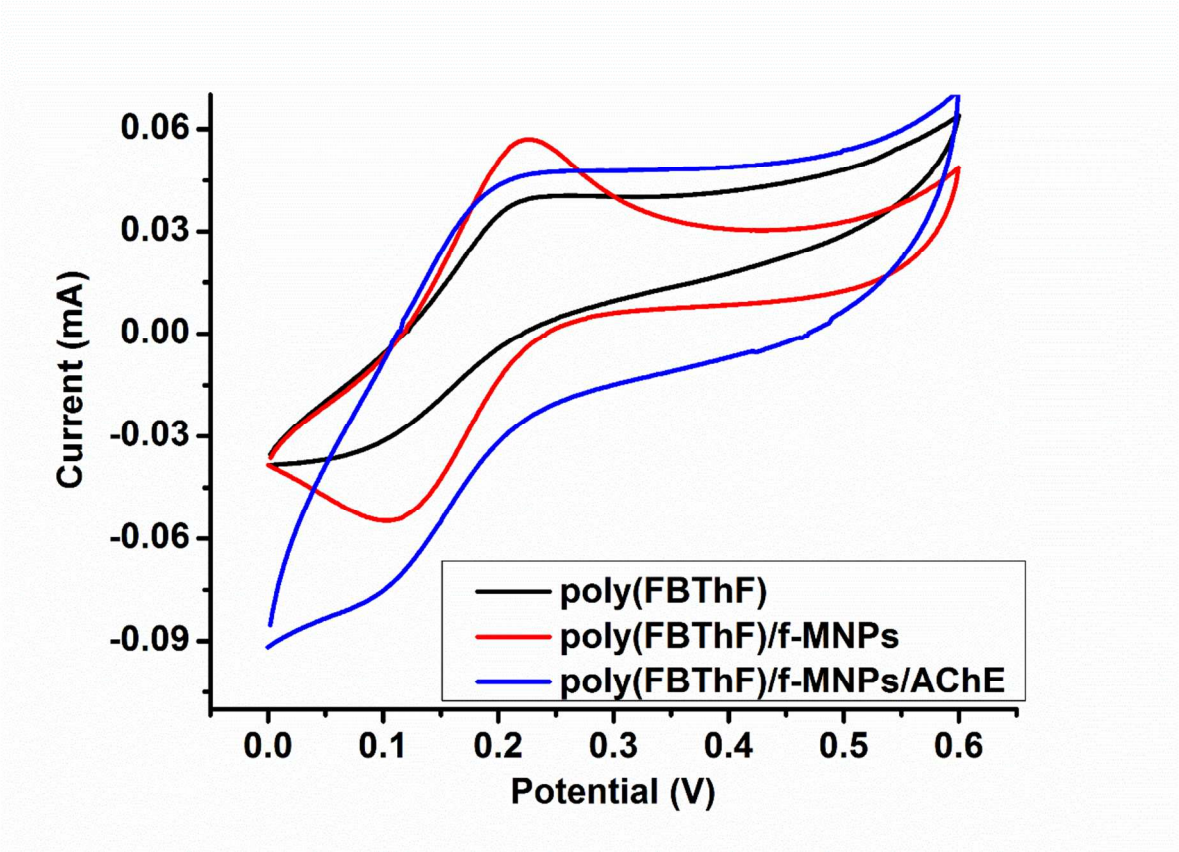


Figure 4. Cyclic voltammograms of poly(FBThF), poly(FBThF)/f-MNPs and poly(FBThF)/f-MNPs/AChE in 5 mM $Fe(CN)_6^{3-/4-}$ containing 0.1 M KCl and pH 7.5 PBS.

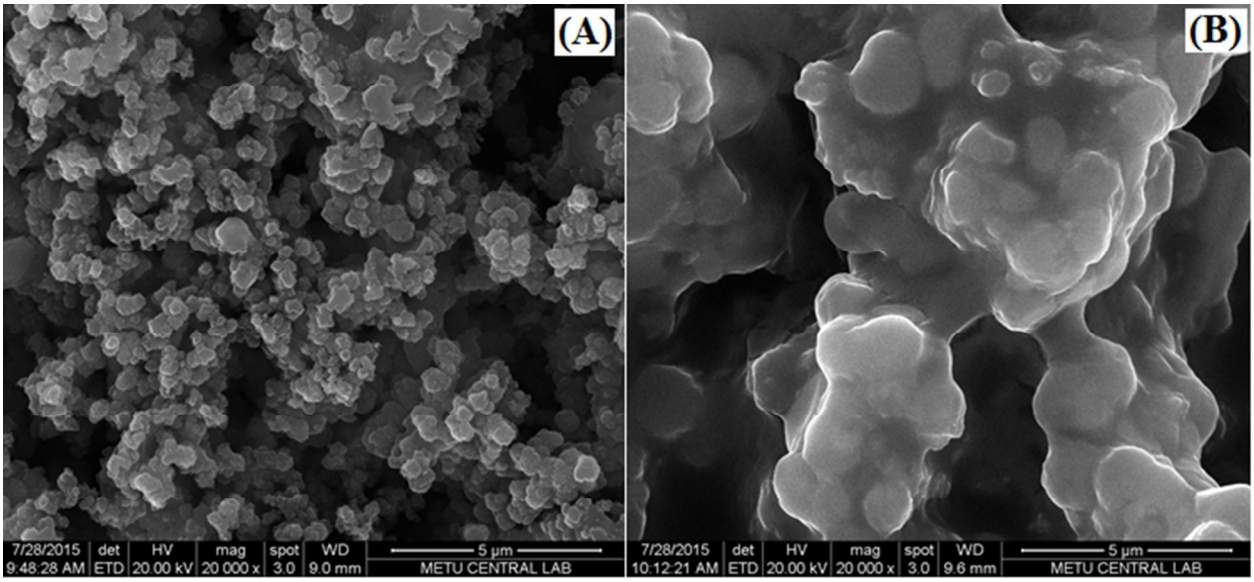


Figure 5. SEM images of **(A)** poly(FBThF), **(B)** poly(FBThF)/f-MNPs/AChE, under optimized conditions.

3.5. Analytical Characterization

The amperometric response of poly(FBThF)/f-MNPs/AChE was investigated as a current vs. time curve where the current changed and reached to a stable value within 5 s upon addition of substrate solution. Fig. 6 displayed the calibration curve for AThCl. A linearity was found between 0.0125 mM and 2.6 mM acetylthiocholine chloride in 50 mM PBS pH 7.5 as given with an equation; $y = 3.0117x + 0.2626$ (R^2=0.990). The limit of detection (LOD) and sensitivity were calculated to be 6.66×10^{-3} mM (S/N=3) and 45.014 µA $mM^{-1}cm^{-2}$, respectively. The sensitivity of the biosensor was significantly improved, compared to previously reported studies (Table 1). The high activity of the sensor can be correlated to the large surface area of the nanomaterial, which is a desired property toward enabling analytes to reach to the diffusion layer of the electrode rapidly. We believe that the tendency of MNPs to adsorb proteins and their ability to

further couple with biomolecules, thanks to the functional groups on MNPs, had a great impact on achieving such remarkable sensitivity.

Table 1. K_M^{app} and sensitivity of reported electrodes and poly(FBThF)/f-MNP modified graphite electrode for AChE

Modifications	Type of electrode	K_M^{app}	Sensitivity	Ref.
Poly(FBThF)/f-MNPs/AChE	Graphite electrode	0.7311 mM	45.014 $\mu AmM^{-1}cm^{-2}$	This work
CS/AChE/PB-CS/ERGO-AuNPs-β-CD/GCE	Glassy carbon electrode	0.106 mM	14.5 and 9.51 μAmM^{-1}	[44]
cGO-NTA-Ni-AChE	Glassy carbon electrode	NR	2.23 μAmM^{-1}	[45]
AChE/CPE	Carbon paste electrode	1.12 mM	0.69 μAmM^{-1}	[46]
f-MWCNT/poly(SNS-NH_2)/AChE	Graphite electrode	1.038 mM	24.16 $\mu AmM^{-1}cm^{-2}$	[2]
AChE-pRGO-CHIT/GCE	Glassy carbon electrode	0.73 mM	NR	[47]
PDDA/AChE/PDDA/CNT/GC	Glassy carbon electrode	1.75 mM	NR	[48]
AChE/hybridpolymer/MWCNT	Platinum electrode	1.15 mM	0.093 $\mu A/\mu M \cdot cm^2$	[49]
AChE-Fe_3O_4NPs/c-MWCNTs	Indium Tin Oxide	NR	0.402 mA/μM	[50]

NR: Not reported

As the substrate concentration increased, a plateau was observed which represents characteristics of Michaelis-Menten kinetics. The apparent constant (K_m^{app}) was determined as 0.7311 mM according to Linewaver-Burk equation.[51] This value was much smaller than that for AChE immobilized on a) hybrid polymer membrane with integrated MWCNT (1.15mM)[49], b) on MWCNT decorated poly(SNS-NH_2) (1.038 mM)[2], c) on CPE (1.12 mM)[46] and PDDA and d) CNT modified GC electrode (1.75 mM)[48] (summarized in Table 1). A small K_m value implies that AChE immobilized on poly(FBThF)/f-MNPs electrode presents higher affinity for AThCl. This can be accounted for a favorable orientation of biomolecule in the sensing design. It also means that AChE immobilized on the electrode can retain its catalytic activity since the combination of the f-MNPs nanostructures and conducting polymer poly(FBThF) creates an outstanding platform for the biomolecule.

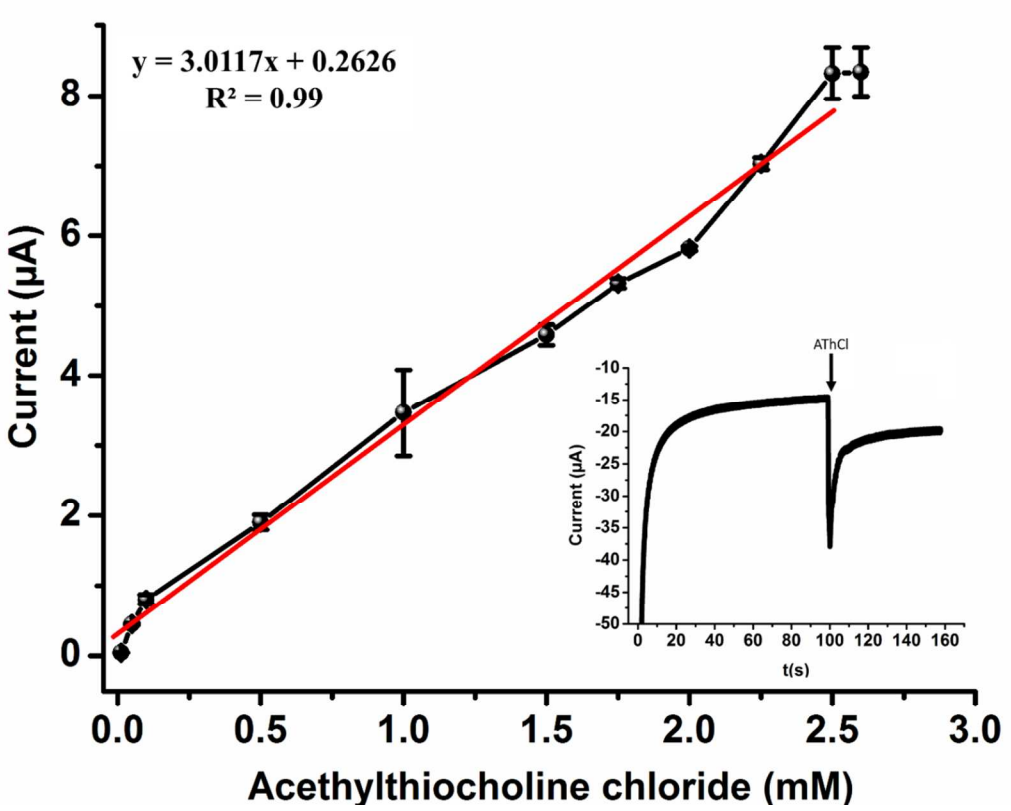


Figure 6. Calibration curve for AThCl (in 50 mM PBS, pH 7.5) (A typical amperometric signal of the biosensor as an inset for 2 mM AThCl before pesticide incubation). Error bars show the standard deviation (SD) of three measurements.

3.6. Application of the Biosensor for Pesticide Determination

Herein, paraoxon and trichlorfon were selected as the model inhibitors to examine pesticide sensitivity to AChE. For this purpose, pre-incubation method was used. Biosensor responses were investigated by incubation in the given concentrations of pesticide solutions. During exposure, pesticides interact with the serine site of AChE. A decrease in the biosensor response via forming phosphorylated adducts is a result of blocking the serine hydroxyl moiety. Such an inhibition caused a decrease in the function of the enzyme.

The incubation time is defined as the reaction time of the enzyme with the inhibitor. Each pesticide was analyzed for their effect on AChE activity at given incubation times (2-20 min) in a pesticide solution of 0.01 μM. Fig. 7A refers to the inhibition degree of the enzyme. It was increased with increasing the incubation period up to 10 min. This is common for irreversible inhibitors like organophosphorus pesticides[52]. The highest degree of inhibition for each pesticide was not 100 %. This is due to the binding equilibrium of pesticides with the enzyme binding sites.[53] Hence, 10 min of incubation time was selected for the detection of pesticides. This value is in accordance with data reported in previous reports.[54-56]

Under the optimized variables, two linear ranges were observed for each pesticide (Fig. 7B-C). The inhibition of paraoxon was proportional to its concentration between 0.05 μg/L - 5 μg/L and 5 μg/L - 9.28 μg/L, with the coefficient of 0.990 and 0.996, respectively. For trichlorfon, the relationships were from 0.05 μg/L to 4.1 μg/L and from 4.1 μg/L to 9 μg/L, with the regression coefficients of 0.997 and 0.998, respectively. Moreover, the detection limits of the present biosensor were found to be 0.022 μg/L for paraoxon and 0.037 μg/L for trichlorfon. The sensing design exhibited lower detection limit compared to ones reported in literature (summarized in Table 2). For instance, LOD of AChE enzyme on a performed cysteamine self-assembled monolayer on gold-screen printed electrode (2 μg/L),[52] carbon paste AChE based biosensor (0.86 μg/L)[55] and polypyrrole entrapped acetylcholinesterase electrode (1.1 μg/L)[54] were significantly higher compared to our biosensor for paraoxon. For the LOD of trichlorfon, AChE sensor using manganese (III) meso-tetraphenylporphyrin (MnTPP) nanoparticles (NPs) was found to be 0.129 μg/L[57], which is favorably comparable to the LOD value found in this work. The superior performance can be attributed to the integration of f-MNPs and conducting polymer, effective

immobilization of AChE, favorable biocompatible environment for AChE and favorable charge transport properties of the biosensor.

Table 2. Comparison of LOD of AChE biosensors for paraoxon and trichlorfon with the present biosensor.

Pesticide	Modifications	LOD	Ref.
Paraoxon			
	Poly(FBThF)/f-MNPs/AChE	0.022 μg/L	This work
	AChE-glutaraldehyde–Cyst–Au-SPE	2 μg/L	[52]
	PPy-AChE-Geltn-Glut/Pt	1.1 μg/L	[54]
	CPE/AChE	0.86 μg/L	[55]
Trichlorfon			
	Poly(FBThF)/f-MNPs/AChE	0.037 μg/L	This work
	AChE-CHIT/MnTPPNP/GC	0.129 μg/L	[57]

In the construction of inhibition type enzyme biosensors, reactivation of the enzyme is a crucial issue. For this reason the reactivation ability of the biosensors were investigated. First different electrodes were prepared and immersed in paraoxon and trichlorfon solutions,

separately. Then these electrodes were rinsed with distilled water and PBS, respectively. Finally, the electrodes were immersed in PBS (50 mM, pH 7.5) for reactivation. It was found that AChE modified electrode inhibited by paraoxon (2.14 μg/L) was able to achieve 89.91 % of its original activity after immersion in PBS (50 mM, pH 7.5) for 4 h. It was shown that for the electrode inhibited by trichlorfon (2.2 μg/L), 84.13 % of original activity can be achieved after reactivation. Our experimental results indicated that PBS (50 mM, pH 7.5) itself played a role as a reagent in the reactivation of AChE. Other researchers have used some other approaches such as simple rinsing and incubating with pyridine 2-aldoxime methochloride (2-PAM)[2,58,59] for the same purpose. When we compared our results with other reactivation agents[58,59], this method was simple and it provides a reusable enzyme electrode.

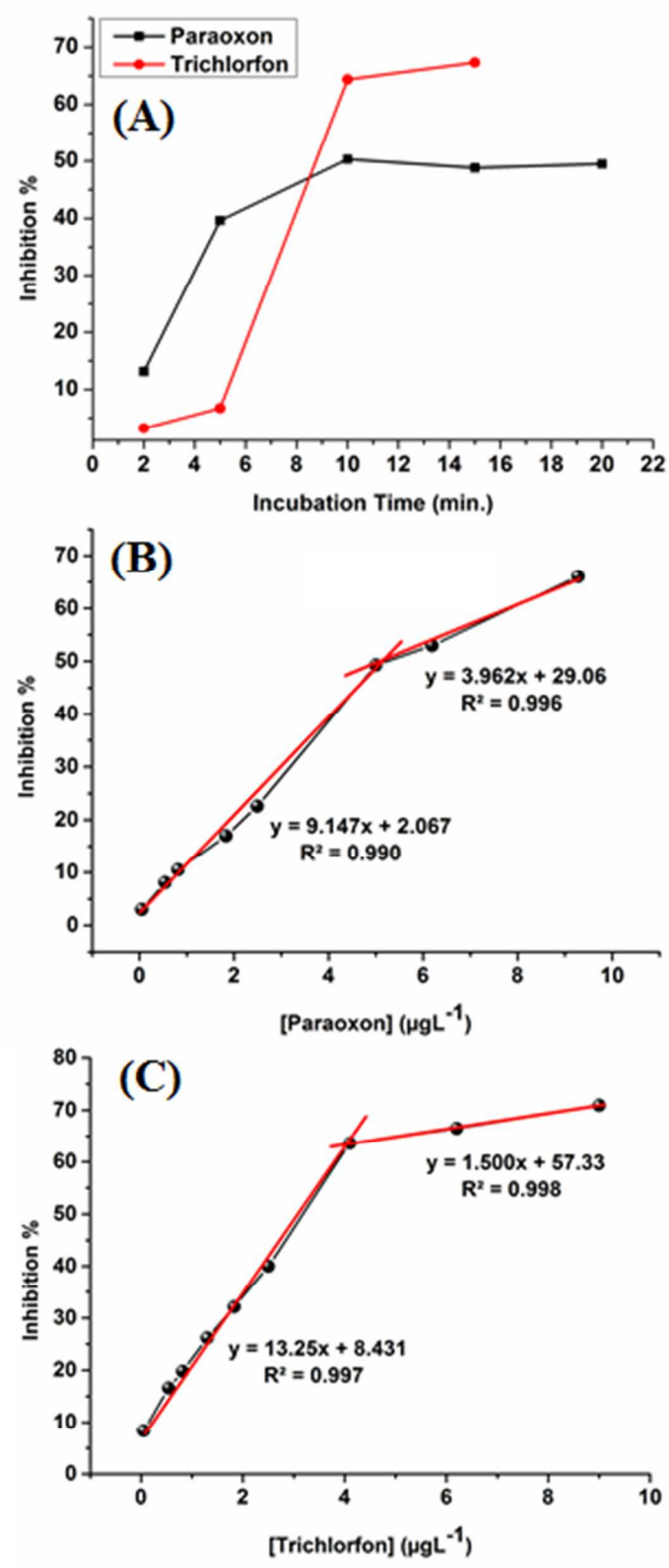

Paraoxon
Trichlorfon
(A)
Inhibition %
Incubation Time (min.)
(B)
y = 3.962x + 29.06
R² = 0.996
y = 9.147x + 2.067
R² = 0.990
Inhibition %
[Paraoxon] (μgL-1)
(C)
y = 1.500x + 57.33
R² = 0.998
y = 13.25x + 8.431
R² = 0.997
Inhibition %
[Trichlorfon] (μgL-1)

Figure 7. (A) Incubation time optimization for two pesticides. **(B-C)** Calibration curves for paraoxon and trichlorfon (in 50 mM PBS, pH 7.5, 2.0 mM AThCl, 10 min incubation time).

3.7. Reproducibility/Stability and Real Sample Analysis

The operational reproducibility of the bio-sensing system was investigated using a freshly prepared poly(FBThF)/f-MNPs/AChE electrode in 2 mM AThCl. The standard deviation (SD) and relative standard deviation (RSD) of the biosensor for 20 measurements with the same sensor were calculated as ± 0.08 and 6.14 %, respectively. Similarly, precision of the poly(FBThF)/f-MNPs/AChE electrode was evaluated by assaying one enzyme electrode for four consecutive studies in 2 mM ATCl after immersed in 1.3 µg/L of paraoxon and trichlorfon for 10 min. The SD and RSD of paraoxon and trichlorfon were found to be ± 0.13, 2.90% and ± 0.10, 2.01%, respectively. These results indicated that the sensor exhibited good reproducibility with acceptable precision for consecutive measurements of the substrate in the presence and absence of the model inhibitors.

In order to evaluate long-term stability, the biosensor responses were recorded during a span of 60 days using 2 mM AThCl solution. It was stored at +4°C. For 10 days no significant activity loss was monitored which reveals the desirable stability of the proposed sensor. After 60 days, an average of 35 % decrease in response was observed. Covalent binding between f-MNPs and the enzyme molecules and use of an excellent support material (CP) were the main reasons for the long-term stability.

The applicability of the proposed biosensor was assessed by the determination of paraoxon and trichlorfon in tap water samples. For this reason, tap water samples were fortified with a solution of each pesticide to obtain concentrations between 0.05 and 0.814 µg/L. No filtration were done prior to use. As shown in Table 3, the recoveries were found to be between 98.1 % and 110.0 %.

The results indicated that the biosensor could be used for the analysis of real samples. Moreover, during the analysis no interference effect was observed since our results are in a good agreement with calculated values.

Table 3. Determination of paraoxon and trichlorfon in tap water samples

Sample (tap water)	Taken (µg/L)	Found (µg/L)	Recovery (%)
Paraoxon			
1	0.050	0.055	110.0
2	0.540	0.580	107.4
3	0.814	0.840	103.2
Trichlorfon			
1	0.050	0.049	99.6
2	0.540	0.53	98.1
3	0.814	0.822	101.1

4. CONCLUSIONS

Herein, functional magnetic nanoparticles decorated with silica and carboxyl groups were synthesized in several steps and characterized using FT-IR and SEM techniques. Then, the monomer, 4,7-di(furan-2-yl)benzo[c][1,2,5]thiadiazole (FBThF) was electrochemically polymerized on the electrode surface. The matrix combined with polymer and functionalized magnetic nanoparticles were successfully tested for bio-sensing applications in the analyses of pesticides. Our results revealed that incorporation of magnetic nanoparticles with polymeric matrix improves the biosensor characteristics and the stability of the matrix. Moreover, kinetic parameters (sensitivity, K_m^{app} and I_{max}) of the developed biofilm demonstrated an enhanced biosensor performance. To best of our knowledge, the biosensor fabricated in this study showed one of the highest sensitivity values among all the conjugated polymer based pesticide biosensor in the literature. The proposed bio-sensing platform showed long shelf-life compared to other reports.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡ These authors contributed equally.

Supporting Information

TEM image for Fe_3O_4 @SiO_2 nanoparticles, FTIR spectra and effect of scan number, pH optimization, enzyme activity and f-MNPs amounts on the biosensor response.

Note

We acknowledge OYP 2015 grant.

ABBREVIATIONS

Conjugated polymers, (CPs); poly(4,7-di(furan-2-yl)benzo[c][1,2,5]thiadiazole),poly(FBThF); functionalized magnetic nanoparticles, (f-MNPs); Acetylcholinesterase, (AChE); Scanning electron microscopy, (SEM); Cyclic Voltammetry, (CV); Dichloromethane, (DCM); acetonitrile, (ACN); Tetrabutylammonium hexafluorophosphate, ($TBAPF_6$); Fe_3O_4 nanoparticles, (MNPs); Silica modified magnetic nanoparticles (SiO_2-MNPs); Carboxyl group functionalized magnetic nanoparticles, (f-MNPs); Nuclear magnetic resonance, (NMR); Phosphate buffer solution, (PBS); Standard deviation, (SD); Relative standard deviation, (RSD); Apparent Michaelis-Menten constant, (K_M^{app}).

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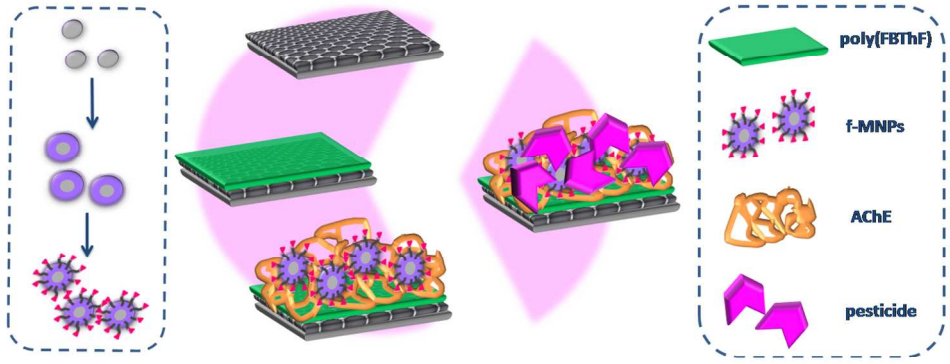

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