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Immobilization of Acetylcholinesterase onto Pyrrole-containing Photocured Thermosets

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Enzyme immobilization,
Pyrrole

Abstract: Acetylcholinesterase (AChE; EC 3.1.1.7) is a group of enzymes that catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh) into choline and acetate. AChE inhibition is commonly utilized as a biomarker for pesticides. In membrane based AChE biosensors the enzyme immobilization onto an electrode surface is of prime importance. In previous studies, conducting polymers-based supports have been used for the immobilization of AChE. In this study, a novel immobilization platform was developed. The simultaneous polymerization of pyrrole and functional thiol/ene monomers was performed to prepare conductive thermosets. AchE was covalently immobilized onto the membranes through the epoxy functional groups. After the immobilization process, the optimal temperature increased to 50 °C, displaying a better thermal stability and the optimum pH was elevated to 8.5. The activity of the immobilized enzyme was tested in the presence of several metals, and it was found that Cu^{2+} ions caused a noticable inhibition. After 10 cycles, the immobilized enzyme retained 51% of its original activity. In accordance with our results; the durability and the stability of the immobilized enzyme were improved. In future studies, the method applied here can be used in the design of an AchE biosensor.

Asetilkolinesterazın Pirol İçeren Fotokürlenmiş Termosetlere İmmobilizasyonu

Anahtar Kelimeler
Asetilkolinesteraz,
Tiyol-en,
Fotokürlenme,
Enzim immobilizasyonu,
Pirol

Öz: Asetilkolinesteraz (AChE; EC 3.1.1.7), nörotransmiter asetilkolinin (ACh) kolin ve asetata hidrolizini katalize eden bir grup enzimdir. AChE inhibisyonu, pestisitler için biyolojik bir belirteç olarak yaygın olarak kullanılmaktadır. Membran temelli AChE biyosensörlerinde, elektrot yüzeyine enzimin immobilizasyonu oldukça önemlidir. AChE'nin immobilizasyonu için iletken polimerlerl de içeren pek çok farklı destek kullanılmıştır. Bu çalışmada, yeni bir immobilizasyon platformu geliştirilmiştir. Pirol ve tiyol-en monomerleri ile birlikte polimerleştirilerek iletken polimerik termosetler elde edilmesi planlanmıştır. AChE epoksi grupları üzerinden membrana immobilize edilmiştir. İmmobilizasyon işleminden sonra enzimin optimum sıcaklığı 50°C'ye çıkarak daha iyi bir termal kararlılık gösterirken, optimum pH değeri 8.5'a yükselmiştir. İmmobilize enzimin aktivitesi üzerine çeşitli metallerin etkisi araştırılmış ve Cu^{+2} iyonlarının önemli bir inhibisyona yol açtığı bulunmuştur. 10 döngüden sonra, immobilize enzimin başlangıç aktivitesinin %51'ine halen sahip olduğu belirlenmiştir. Sonuçlarımızla uyumlu olarak immobilize edilen enzimin kararlılığının ve dayanıklılığının arttığı görülmektedir. Gelecek çalışmalarda burada önerilen metod bir AChE biyosensör tasarımında kullanılabilir.

1. Introduction

Acetylcholinesterase (AChE; EC 3.1.1.7) is a hydrolase that is vital for living organisms [1, 2]. It converts the neurotransmitter acetylcholine to choline and acetate. AChE terminates impulse transmission by rapidly hydrolyzing acetylcholine (ACh) [3]. Abrupt inhibition of AChE catalytic activity leads to excess acetylcholine at neuromuscular synapses and causes muscle paralysis and contractions, severe respiratory dysfunction, and death [4].

Highly toxic AChE-inhibiting organophosphates (OPs) and carbamates (CMs) are mostly used pesticides throughout the world. Pesticides, can pass into the air, water, and soil, and from there to other creatures

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living in these environments, and accumulate in the adipose tissue of humans and animals. Inhibition of the AChE enzyme is used to detect pesticides in environmental and food pollution. AChE can be used for the fabrication of biosensors [5]. Most AChE sensors designed for practical applications use immobilized enzymes. AChE is not only used for biosensing features it is also used for diagnosis of neurodegenerative diseases [6, 7]. Nevertheless, it can be used as a biocatalyst in organic synthesis [8].

Enzyme immobilization is the physical or chemical confinement of enzymes within or onto a support material. Enzyme immobilization has several advantages. Enzymes can be used over and over again, which is especially important for enzymes that are difficult and expensive to produce. As the enzyme is retained in the matrix, the product is not contaminated with the enzyme, and since the matrix protects the enzyme as a physical barrier, the enzyme becomes resistant to influences such as extreme pH and temperature. Immobilized enzymes can be controlled much more easily [9,10]. AchE was immobilized onto different substrates for pesticide detection [11-15].

In this study, a fast, simple, and single-step method has been aimed to prepare a novel platform for enzyme immobilization. First, allyl glycidyl ether (AGE), 1,3,5-Triallyl-1,3,5-triazine-2,4,6(1H,3H,5H)-trione (TTT), pentaerythritol tetrakis(3-mercapto propionate) (4SH), photoinitiators, and pyrrole (Py) were blended in definite ratios and then subjected to polymerization under UV light. Polypyrrole is an intrinsically conducting organic polymer and it was previously shown that Py can be polymerized photochemically by using a cationic photoinitiator or other agents such as $AgNO_3$ [16-20]. The polymerization mechanism of Py in the presence of a cationic photoinitiator is given in the literature [20]. In our system while thiol-ene photopolymerization takes place, pyrrole polymerizes simultaneously. The AGE which is a bifunctional monomer can also participate in the thiol-ene photopolymerization as well as it can polymerize through its epoxy groups and also the thiol groups can participate and react with the epoxy groups. The originality of this work arises from the combination of these multi-curing mechanisms in one material design for the first time. The combination of different polymerization mechanisms in one-step, and the incorporation of light-triggered polymerization are the novel, original and intriguing sides of this study.

AchE was immobilized onto the prepared thiol-ene photocured films through the epoxy functional groups (the remaining unpolymerized epoxy groups in the polymer matrix). Furthermore, a second route for the immobilization was performed by, modifiying the free epoxy groups which did not undergo polymerization. The modification was perfomed by reacting the epoxy groups with polyethyleneimine (PEI). Then, the amine functionalized discs were further reacted with glutaraldehyde and the enzyme was attached. Prepared films were characterized before and after the immobilization process and hydrolytic response towards acetylthiocholine iodide was assessed using a spectrophotometric bioassay. This study focuses on the material preparation and enzyme immobilization performance rather than the conductivity measurements and sensor applications which will be the subject of another paper in the future.

2. Material and Method

All chemicals including Acetylcholinesterase from Electrophorus electricus were of high purity and maintained from Sigma Aldrich Chemical Co. (St.Louis, USA). Pyrrole was distilled before use.

2.1. Structural characterization of polymeric support

Fourier Transform Infrared Spectroscopy (FTIR) measurements were performed by using Perkin–Elmer Spectrum 100 ATR-FTIR.

2.2. Photocured support fabrication

In order to prepare the support material, first 4SH, TTT, AGE, and pyrrole were mixed according to the ratios given below (Table 1) in a beaker. The ene:thiol ratio was kept as 1:1. 4% cationic, and free radical photoinitiator were then added to this mixture. Finally, the mixtures were placed into circular Teflon molds (6 mm diameter) and held under UV irradiation for 10 minutes. The structure of all the monomers are presented in Figure 1. For the enzyme immobilization, TE-APCR encoded films were used. Other compositions were only prepared to understand the effect of cationic photoinitiator, and Py.

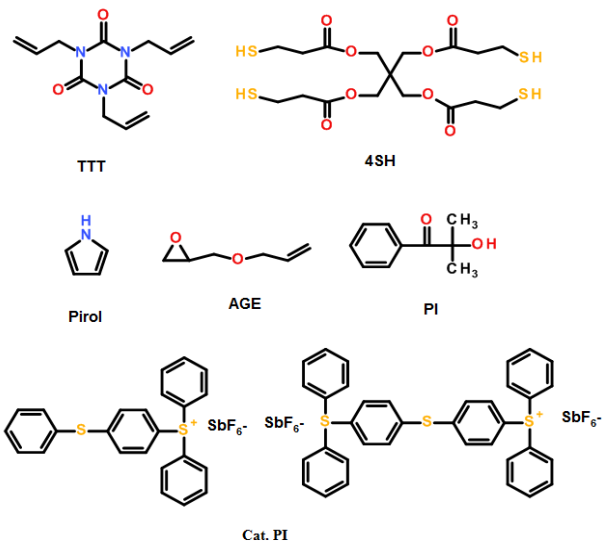


Figure 1. Main chemical compounds used for the synthesis of the polymeric discs.

Table 1. Composition of the photocurable formulations. *

	TE-APCR	TE-ACR (Py-free)	TE-APR (Cat. PI-free)
AGE**	5	5	5
TTT**	5	5	5
4SH**	5	5	5
Pyrrole	10%	-	10%
Cat. PI	4%	4%	-
PI	4%	4%	4%

*TE:Thiol-ene, P:Py, C:cationic photoinitiatior, R: free radical photoinitior. 10% Py and 4% of each initiator were added. ** mmoles

2.3. Immobilization of AChE onto the prepared support

For the immobilization studies we used the TE-APCR films. AChE was immobilized via two different ways. In the first method, polymeric discs were added to phosphate buffer (10mL, pH 7.0) containing a 63 µg of AChE. Films were incubated for 24 hours in an orbital shaker. After this period, films were removed from enzyme solution and the covalently immobilized enzyme was determined according to the Bradford method as described elsewhere [21, 22].

For the second immobilization route, polymeric discs were kept in an aqueous solution of PEI. The solution was prepared by transferring 1 gram of 30% stock solution of PEI to 30 mL of water. The films were kept in this solution for 24 hours. The solution was heated in a water bath to 40°C during this procedure.

After that the films were removed, washed with distilled water and dried. Then, the films were immersed into a 25% glutaraldehyde solution for 1 hour. The activated discs were removed and then AChE was immobilized as described previously.

2.4. AChE activity assay

The enzymatic activity was determined according to the Ellman method [23]. Acetylthicholine iodide (ATChI) was used as substrate and 5,5'dithiobis-(2-nitrobenzoic acid) (DTNB). The details of this procedure can be found in our previous paper [22].

2.5. Effect of pH and temperature

The influence of pH on the activity of immobilized and free AChE was investigated in a pH range of 7.0 to 9.0. For this purpose phosphate buffers (50 mM) at different pH values were prepared. The effect of temperature on the enzymatic activity was also performed in the temperature range of 25°C-55°C. Details of these measurements can be found in the literature [22, 24].

2.6. Determination of Kinetic Parameters

The maximum reaction velocity (*V*max) and Michaelis constant (*K*m) parameters were calculated from Lineweaver–Burk plot at optimum pH and temperature. The activities of the immobilized and free enzymes were determined at different substrate concentrations (0.1-2 mM).

2.7. Determination of the re-use and storage values

To the pre-weighed immobilized enzyme, 100 µL of 2 mM acetylthiocholine iodide in distilled water, 100 µL of DTNB solution (10 mM in phosphate buffer solution), and 1.8 mL of phosphate buffer solution were added. The mixture was incubated for 10 minutes at 37°C. Then the immobilized enzyme was taken out of the solution and the absorbance of the solution at 412 nm was measured. The immobilized enzyme was added to a new substrate solution to start a new cycle. For details see references 16 and 18.

To demonstate the storage performance of the immobilized and free enzymes, their activities were periodically measured at their optimum pH and temperature conditions for two months. During this time period, samples were kept at 4°C.

2.8. The effect of metal ions

Aqueous solutions (0.1 mM) of different metals were prepared (Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}). The effect of these cations on the enzymatic activity of the immobilized and free AchE was investigated.

2.9. Effect of pesticides

0.01µg of the pesticide (azinphos methyl, chlorpyrifos-ethyl, or cyanofenphos) were dissolved in 10µL acetonitrile. Then the inhibitory effect of these pesticides on the immobilized and free enzymes was determined under optimum conditions.

3. Results

In this work, thiol-ene photopolymerization and photoinduced pyrrole polymerization techniques were combined with the aim to develop an immobilization support material that can be prepared quickly and easily and can also be suitable for sensor applications.

To verify the polymerization of Py within the prepared systems, Py-free and cationic photoinitiator-free formulations were prepared. The photographs of the fabricated films are given in Figure 2. The films of Py-free formulation were transparent while the sample TE-APCR encoded samples which contain pyrrole, AGE and cationic and radical photoinitiators were brown. This color change is a direct evidence of polypyrrole formation. Generally Py turns to black when polymerized, thus it can be said that Py was partially polymerized in the formulation. When cationic photoinitiator was not used, the films displayed a yellowish color. The structural characterization of the photocured thermoset materials was conducted by recording their FTIR spectra.

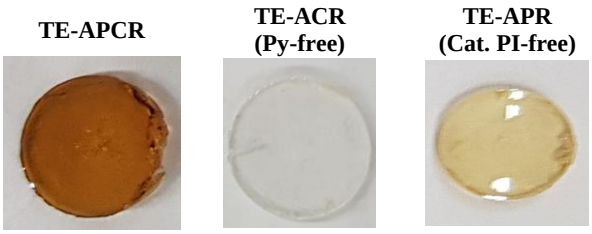


Figure 2. Digital photographs of the photocured discs.

The FTIR spectra of the photocured TE-APCR and the precursor monomers; AGE, 4SH, TTT and pyrole can be seen in Figure 3. The vibration band at 911 cm^{-1} for TE-APCR is due to AGE's epoxy ring which reveals that not all epoxy groups are polymerized [25]. We take the advantage of this feature and immobilized enzymes through these epoxy groups. The vibration bands at 1732 and 1680 cm^{-1} belong to carbonyl groups of 4SH and TTT, respectively. The absence of SH and allyl bands at 2550 cm^{-1} and 1645 cm^{-1}, respectively prove that the polymerization was achieved and the polymeric films were prepared succesfully (FTIR spectrum of TE-APCR). The vibration band appeared at 1794 cm^{-1} is due to propylene carbonate (cationic photoinitiator) and the bands at 2980-2800 cm^{-1} belong to aliphatic $-CH_2-$ and $-CH_3$ groups. Since the bands of polypyrrole are screened by the bands of thiol-ene monomers, they cannot be clearly seen but the vibration band at 3397cm^{-1} can be attributed to -NH stretching of polypyrrole. Figure 4 displays the FTIR spectrum of PEI modified films. It can be seen in this spectrum that all the epoxy bands have disappeared and the band around 3500 cm^{-1} belongs to -OH and amine groups.

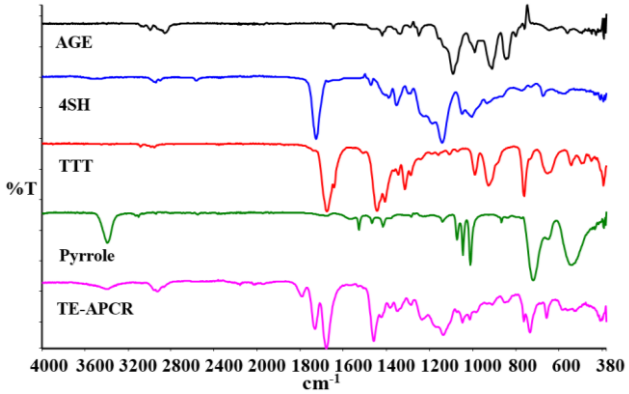


Figure 3. FTIR spectra of AGE, 4SH, TTT, Py and TE-APCR encoded films.

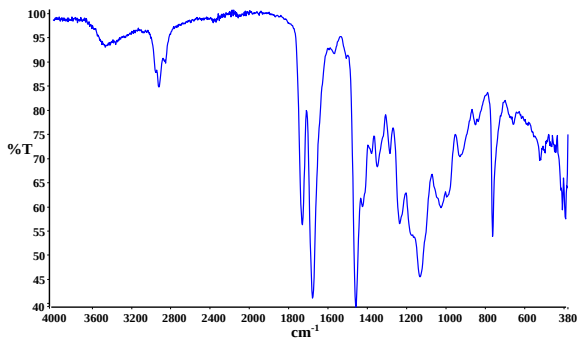


Figure 4. FTIR spectrum of PEI modified TE-APCR films.

AChE immobilization is an intruging area of research for the fact that immobilization can lead to improved properties such as shelf-life, re-usability, pH and thermal stability which are all beneficial and required for the development of biosensors with enhanced features. AChE was covalently immobilized onto the epoxy-functional TE-APCR and PEI-modified TE-APCR films successfully (Scheme 1). The amount of covalently immobilized AChE was calculated on per gram of TE-APCR and PEI-modified TE-APCR was found as 60.27 μg and 54.82 μg with high binding capacities of 95.66% and 87.01% respectively.

The influence of pH around 7.0-9.0 was investigated. The results of pH effect are presented in Figure 5 as relative activity vs. pH. The maximal enzyme activity was detected at pH 8.0 for the free enzyme while, the same parameter was determined as 8.5 for the immobilized enzymes. The impact of temperature on the activity of immobilized and free AChE was examined by altering the temperature from 25-55 °C and the relative enzyme activity versus tempertaure graph was shown in Figure 6. As it is seen from Figure 6, free enzyme displayed its maximum activity at around 30°C. The thermal stability for the TE-APCR and PEI-modified films increased up to 50 °C.

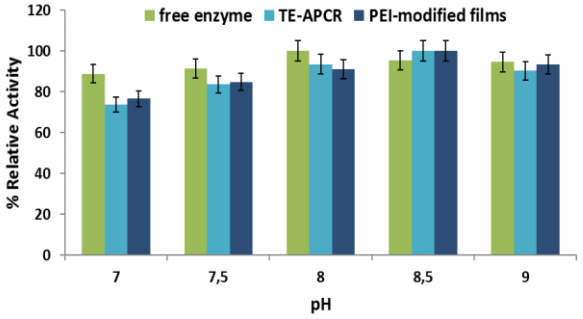


Figure 5. pH versus relative activity plot.

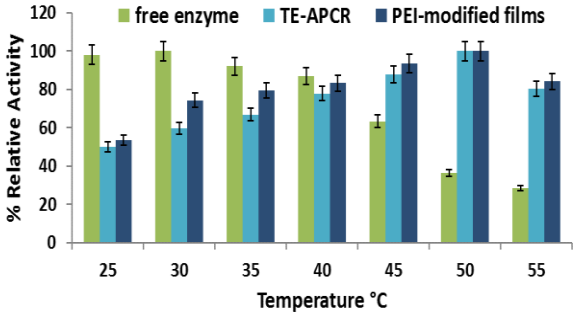
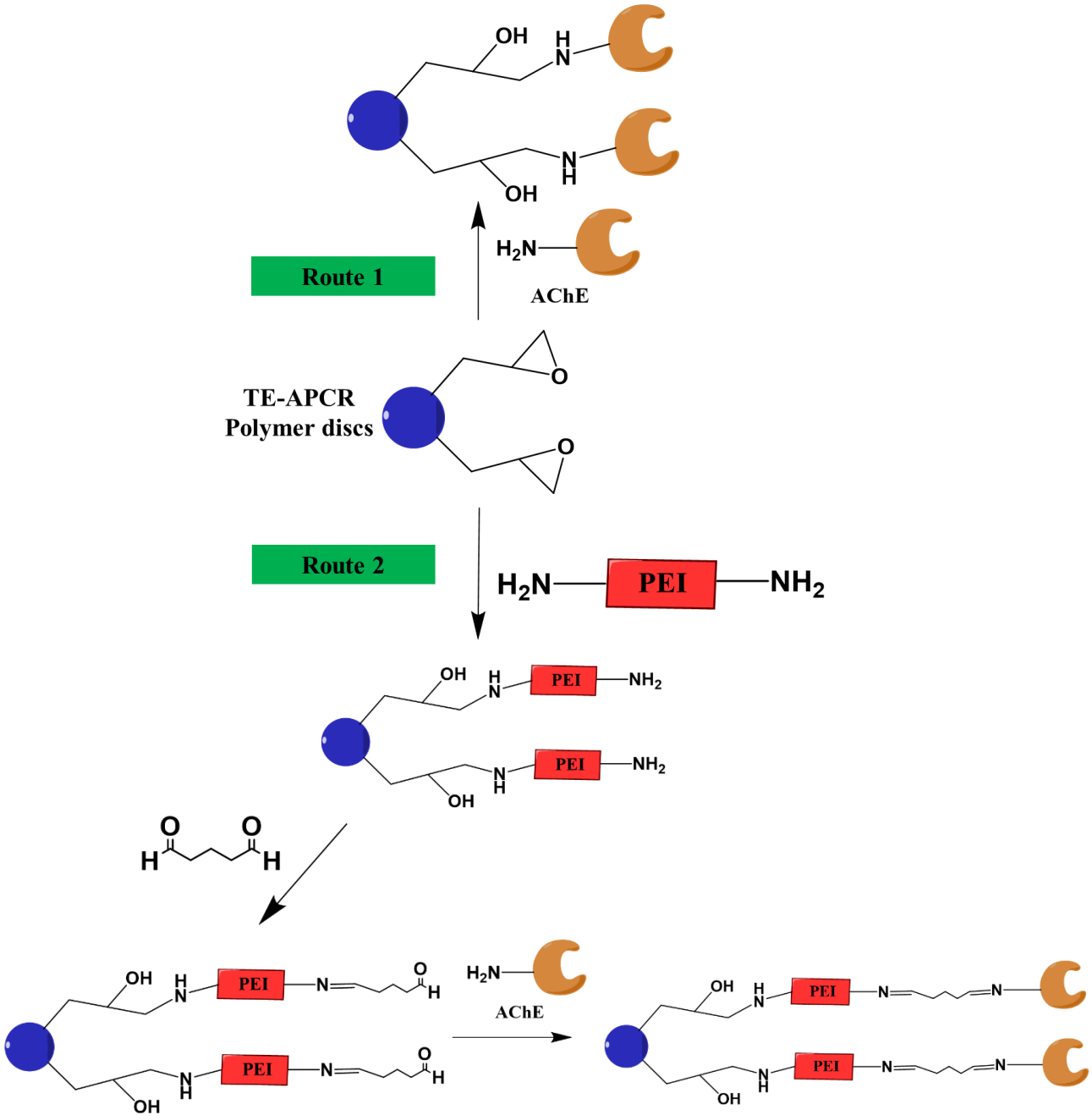


Figure 6. Temperature versus activity plot.

*K*m and *V*max were evaluated from the Lineweaver–Burk plot for immobilized and free AChE, at the optimum pH and temperature values, in certain concentration range (0.01-1.5 mM) of ATChI solution. The measured *K*m and *V*max values are given in Table 2. The immobilized AChE possess a lower *K*m and *V*max values compared to free AChE. TE-APCR immobilized enzymes did not display significantly higher activity when compared to PEI-modified films.



Scheme 1. Depiction of the immobilization routes applied in this work.

Table 2. Kinetic values of the immobilized and free enzymes

	Free enzyme	TE-APCR	PEI-modified films
***Km* (mM)**	1.25	0.182	0.416
***Vmax* (mM/min)**	1.53	0.5	0.018

The adequate reusability of immobilized enzymes is a prerequisite for different enzymatic applications. According to the re-use tests, the residual activity of the immobilized AChE was declined with the increasing number of cycles (Figure 7). Nonetheless, after the 10th cycle, the remaining activity of the TE-APCR and PEI-modified films were 74 and 67 % of their initial activities, indicating a good operational stabilities for the enzyme-based applications.

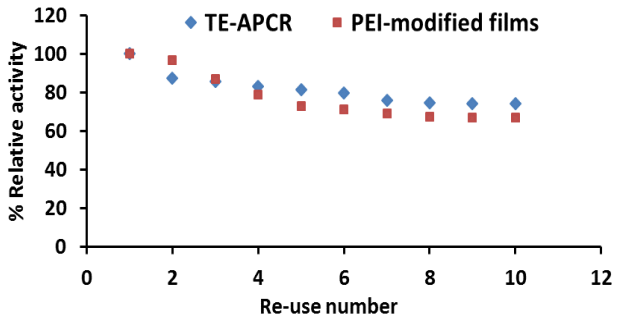


Figure 7. The re-use performance of the immobilized enzymes.

Generally, enzymes lose their activities during storage. The plot of the residual activity percentage versus time is given in Figure 8. Contrast to free enzyme

which lost its activity completely within 60 days, TE-APCR and PEI-modified films retained 66.9% and 69.9 % of their initial activities under the same storage time.

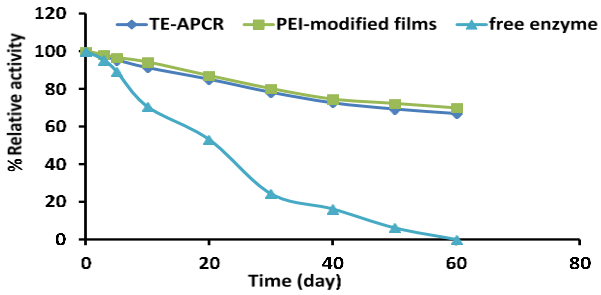


Figure 8. Relative activity change versus time plot for the immobilized and free enzymes.

The effect of Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} cations on the enzymatic activity is demonstrated in Figure 9. Remaining activity can be sorted as Mn>Mg>Ca>Zn>Cu for the free enzyme. Free enzyme displayed a remaining activity of 33% and 32% in the presence of Mn and Mg ions while displayed the least activity for Cu (3.14%). The TE-APCR and PEI-modified films exhibited very similar remaing activities, therefore only the results for TE-APCR are given. The remaining % activity can be sorted as Cu>Ca>Zn>Mg>Mn for TE-APCR.

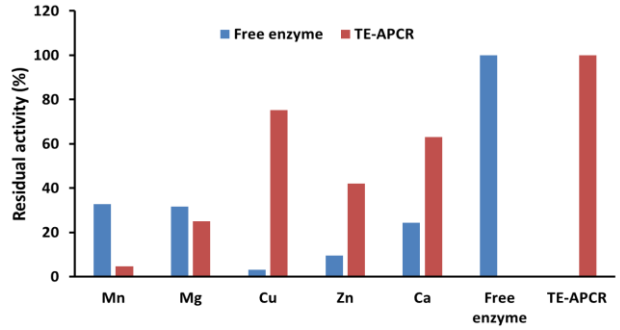


Figure 9. The effect of different metal cations.

The inhibitory activity of pesticides (azinphos methyl, chlorpyrifos-ethyl, or cyanofenphos) and acetonitrile (solvent) were investigated for free enzyme and immobilized enzymes (Figure 10). It can be seen from the results that the activity of both the immobilized and free enzymes declined in the presence of the pesticides. However, the decrease in the activity is much more pronounced in immobilized enzymes.

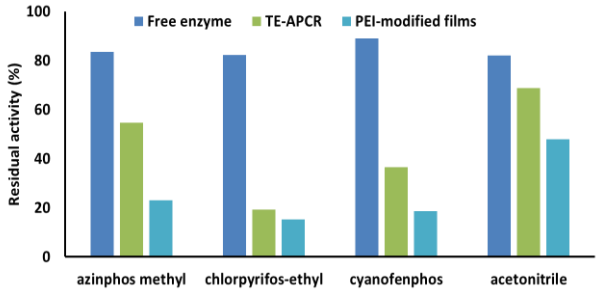


Figure 10. The effect of different pesticides on enzyme activity.

4. Discussion and Conclusion

In the literature, there are various levels of loading values for AChE onto different polymeric matrices. Immobilization efficiency changes according to the polymer type and reaction conditions. In this study, AchE was immobilized onto Py-containing photocured films (TE-APCR). The prepared films displayed excellent binding capacity percentages for AChE. Among the routes applied for immobilization, the first route involves the immobilization of the enzyme by reacting with the epoxy groups on the surface of the films. Due steric and diffusional limits, it can be stated that both physical adsorption and covalent attachement were responsible for the immobilization. In the second route, enzyme was covalently attached to the surface of the films with the aid of PEI and glutaraldehyde. The optimum pH slightly shifted to higher pH values in this work. This slight shift to basic range after covalent immobilization was reported previosuly in literature and can be attributed to the surface charges of the polymer support and also to the change of AchE conformation [26-28].

The covalent immobilization is known to render the enzyme suitable for high temperature applications. Here, this fact was also observed and the maximum temperature that the enzyme is active was shifted to as high as 50 °C. Saleem et al., immobilized AChE onto porous silicon wafers and found that the thermal stability could be increased up to 90°C by immobilization [29]. The increase in the thermal stability of the immobilized enzyme results from the protection of the active site of the enzyme upon immobilization. The deformation and damage of the enzyme with temperature is prevented by the conformational change. Besides the mobility of the enzyme is restricted and it becomes stable. Hence, the immobilized enzyme exhibits greater activity at higher temperatures compared to free enzyme [30].

The decrease in the *K*m value after immobilization implies that the tendency of the enzyme to its substrate is increased. The kinetic parameters are affected from the changes in the conformational structure of the immobilized enzyme, steric hindrance, and diffusion effects [31]. Similar to this work, when AChE was immobilized on polyacrylic acid-based nanofibers the *K*m value was found as 0.5008 mM [22]. In other works comparable low Km values were also reported [32-33].

Here, the immobilization of the enzyme on the newly developed support material significantly improved both the re-use and storage stability. In two seperate studies, Stoilova et al., found that the immobilized enzyme showed 55% activity and 35% after 10 cycles [26, 27]. Gabrovska et al., immobilized AChE onto modified acrylonitrile copolymer membranes and found that immobilized enzyme retained 50% of its original activity after 10 cycles [34]. Compared to

these studies, our support material is superior interms of reusability. The storage stability of our support material is superior or alike compared to the AChE immobilized on different substrates [22, 27, 34, 35].

When the results of the effects of the metal cations on the activity of the free and immobilized enzyme it is clear that the immobilization significantly protected the enzyme against the activity-reducing effects of metal ions. The presence of the pesticides decreased the enzyme activity for the immobilized films. It is known that pesticides inhibit the activity of enzymes by interacting with the hydroxyl group of the serine amino acid in the active site of the AChE [36].

Finally, it can be said that a novel platform for enzyme immobilization was developed in this work. The immobilized enzyme displayed good good thermal and storage stability as well as resistance to metal cations. We plan to use this newly developed polymer support material for biosensor construction in future.

Acknowledgment

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Declaration of Ethical Code

In this study, we undertake that all the rules required to be followed within the scope of the "Higher Education Institutions Scientific Research and Publication Ethics Directive" are complied with, and that none of the actions stated under the heading "Actions Against Scientific Research and Publication Ethics" are not carried out.

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