

Enhancement of Catalytic Stability and Kinetic Evaluation of Acetylcholinesterase Immobilized on Functionalized Fe₃O₄ Magnetic Nanoparticles

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In this study, Fe₃O₄ magnetic nanoparticles (MNPs) were synthesized, and the acetylcholinesterase (AChE) enzyme was immobilized on the functionalized MNPs using the cross-linker glutaraldehyde. The immobilized enzyme (Fe₃O₄@SiO₂-NH₂/GA/AChE) was characterized by scanning electron microscopy-energy scattering spectroscopy (SEM-EDX) and FTIR. Activity for optimum pH was determined in the range of pH 5–11 for free and immobilized enzyme, and the highest activity was observed at pH 8 for free AChE and pH 10 for immobilized AChE. When the kinetic parameters were examined, the K_m and V_{max} values of immobilized AChE were 0.446 mM and 0.659 EU mL⁻¹, respectively, while those of free AChE were 0.163 mM and 0.965 EU mL⁻¹, respectively. The binding affinity of the tacrine inhibitor to the enzyme was lower for the immobilized enzyme than for the free enzyme. Storage stability tests showed that immobilized AChE stored at 4 °C for 60 days retained 95.2% of its activity, while the free enzyme lost 50.4 % of its activity. Furthermore, reuse tests performed for 20 replicates showed that immobilized AChE retained more than 95% of its initial activity. Therefore, the high stability and reusability of the enzyme as a result of the immobilization process may contribute to future studies in terms of biotechnological applications.

1. Introduction

The research on insoluble enzymes and their usage in different applications has been very popular. Since the 1960s, studies on insoluble enzymes and their applications in various fields have gained significant interest. Compared to free enzymes, immobilized enzymes offer several advantages in industrial applications. They exhibit enhanced stability against environmental factors and prolonged usage, facilitate the production of highly pure products, and allow repeated use, particularly in medical applications, where reusability contributes to cost efficiency.^[1–6]

The widespread use of pesticides in agricultural production poses serious environmental and health risks. For this reason, various biotechnological methods have been developed to minimize the toxic effects of pesticides and ensure their rapid detection. Enzyme immobilization techniques play a crucial role in pesticide analysis, with the immobilization of the acetylcholinesterase (AChE) enzyme being particularly preferred in biosensor technologies.^[7,8] The AChE enzyme plays a critical role in detecting the inhibitory effect of organophosphate- and carbamate-based pesticides. Irreversible modification of the catalytic serine residue in the active site of the enzyme by inhibitors leads to inhibition of neurotransduction, which is advantageous in biosensor applications.^[9,10]

Immobilization of enzymes offers advantages such as long-term use and reusability by increasing storage stability. Immobilization can be performed using various organic and inorganic support materials.^[11,12] There are many physical and chemical techniques for enzyme immobilization. Among these techniques, cross-linking and covalent methods are widely used to prevent enzyme leakage from the solution medium. Among the primary techniques employed for enzyme immobilization are physical adsorption onto functionalized surfaces, covalent attachment facilitated by cross-linkers such as glutaraldehyde, and entrapment within carrier materials. Covalent immobilization can occur either directly between the enzyme and the support surface or indirectly through the use of bifunctional cross-linking agents, enabling stable and durable enzyme-support interactions.^[13,14] Magnetic nanoparticles (MNPs) stand out with their low toxicity, high surface area, easily modifiable chemical surfaces, and high enzyme loading capacity. Thanks to these properties, they are considered as a suitable carrier system for enzyme immobilization and have the potential to be used in various fields such as biosensors, biomedical applications, and the food industry. Thanks to the magnetic properties of MNPs, immobilized enzymes can be easily recovered and separated from the reaction medium, which provides cost-effectiveness in industrial processes.^[15,16] Fe₃O₄ MNPs are one of the most widely used carrier materials due to their high chemical and mechanical resistance, biocompatibility, and finely tunable surface properties. With appropriate surface modifications, these nanoparticles can increase the stability and catalytic efficiency of enzymes.^[17] In the literature, it is reported that Fe₃O₄-based nanoparticles are successfully used in enzyme immobilization and maintain their stability under different conditions. In addition, these nanopar-

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ticles have been the subject of research in many fields, such as catalysis, optoelectronics, solar cells, and biosensors.^[16,18]

In light of the growing need for stable and reusable enzyme systems in analytical and biotechnological applications, this study hypothesizes that immobilization of the AChE on functionalized Fe₃O₄ magnetic nanoparticles via glutaraldehyde cross-linking will enhance the stability, reusability, and operational performance of the enzyme by minimizing losses in its catalytic efficiency. Although various supports and immobilization strategies have been reported in the literature, the immobilization of AChE on functional MNPs with high physical stability and many functional properties is still limited. The aim of this study was to synthesize and characterize glutaraldehyde-bound AChE-functionalized MNPs to contribute to potential applications in biotechnological fields. Furthermore, to evaluate free and immobilized AChE in terms of kinetic parameters, thermal stability, long-term storage stability, and reuse performance.

In this study, functionalized MNPs were synthesized by immobilizing the AChE enzyme on the synthesized MNPs and characterized using Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), and energy-dispersive X-ray spectroscopy (EDX). The AChE enzyme was covalently linked to the surface of functionalized MNPs with glutaraldehyde (GA) cross-linker. Thermal and pH stability, storage and operational stability, and kinetic parameters of AChE enzyme immobilized on functionalized MNPs were reported in comparison with the free enzyme. The catalytic performances of the immobilized and free enzymes were evaluated under optimum conditions.

2. Experimental Section

2.1. Chemical Reagents

Glutaraldehyde (25%), APTES (3-aminopropyltriethoxysilane), tetra orthosilicate (TEOS), acetylcholinesterase (AChE), acetylthiocholine iodide (ATCh), iron(II) chloride tetrahydrate (99.0%), iron(III) chloride hexahydrate (99.0%), monobasic sodium phosphate, disodium phosphate, trizma hydrochloride, sodium hydroxide, hydrochloric acid, and tacrine were acquired from the Sigma-Aldrich.

2.2. Synthesis of Magnetic Nanoparticles

Fe₃O₄ magnetic nanoparticles (MNPs) were synthesized in an open-air environment using the co-precipitation method.^[19,20] The system used for co-precipitation consists of a mechanical stirrer operating in the stirring range of 0–2000 rpm, a glass stirring tip, a 250 mL two-necked flask, and stabilizing feet for the flask. Commercially purchased iron (II) chloride tetrahydrate (7.5 g, 99.0%) and iron (III) chloride hexahydrate (10.1 g, 99.0%) were dissolved in 25 mL of water and mixed in a double-necked flask. Then, 25 mL of NH₄OH (25%) was added to this solution and mixed at 700 rpm for 5 min on a mechanical stirrer. It was observed that the color of the solution turned black when ammonium hydroxide was added and the MNPs were synthesized. For characterization, some of these MNPs were precipitated under a magnet, washed four times with distilled water, dried at 45 °C, and powdered.^[19,21]

2.3. Functionalization of Magnetic Nanoparticles

For the preparation of silicon oxide-coated MNPs, MNPs were dispersed in 100 mL of 20% v/v ethanol solution by sonication for 10 min. Then, 10% NH₃ was added. The prepared solution was vigorously stirred, and 5 mL TEOS (99.0%) was added and incubated at 40 °C, 80 rpm for 12 h. MNPs were separated with a magnet and then washed with methanol. Next, APTES was added to the TEOS-coated MNPs dispersed in ethanol, and the final solution concentration was adjusted to 20% w/v. It was then sonicated for 10 min and incubated at 60 °C for 12 h. The particles were separated with a magnet and washed 3 times with methanol. Finally, it was dried at 50 °C for 18 h.^[22,23]

Commercially purchased AChE enzyme was covalently immobilized on the surface of functionalized MNPs using the GA cross-linking agent. 10 mg of the functionalized MNPs were suspended in 1 mL of GA (25%) solution prepared in distilled water and incubated at 25 °C with stirring (130 rpm) for 2 h. The resulting GA-activated MNPs were separated from the solution using a magnet and washed with distilled water to remove the excess the GA. The functionalized support material was then suspended in 100 µL of AChE solution in phosphate buffer (50 mM, pH 7.6) and incubated at 25 °C with gentle shaking for a while. After incubation, immobilized AChE-bound MNPs were detached with a magnet and washed three times with phosphate buffer (50 mM, pH 7.6) to remove unbound AChE and stored at 4 °C for analysis.^[24] The percentage of immobilization was determined from the change in enzyme activity in the solution before and after immobilization.

$$\text{Immobilization yield (\%)} = X / ((A - B)) \times 100 \quad (1)$$

where A = added enzyme, B = free enzyme, and X = immobilized enzyme.

2.4. Characterization

Spectral data measurements for characterization were performed at Bilecik Seyh Edebali University, Central Research Laboratory. The FTIR spectrum was captured using a PerkinElmer/Spectrum 100 spectrometer, covering wavenumbers between 400 and 4000 cm⁻¹. Elemental properties were determined by EDX. SEM-EDX analysis was performed using a ZEISS/Supra 40 VP scanning electron microscope equipped with an EDX apparatus.

2.5. Enzyme Activity

The modified Ellman spectrophotometric method was employed to evaluate AChE enzyme activity.^[25,26] In this procedure, 0.5 mg of functionalized MNPs immobilized AChE (Fe₃O₄@TEOS/APTES/GA/AChE) was dispersed in 4 mL of trizmahydrochloride buffer solution (0.1 M, pH = 8) and stirred for 5 min. Subsequently, 50 µL of acetylthiocholine iodide (ATChI, 10 mM) and 50 µL of 10 mM DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) were added to the mixture. Activity measurements of enzyme-bound and non-enzyme-bound MNPs were performed. In the Ellman method, thiocholine, produced by the enzymatic hydrolysis of the substrate, reacts with DTNB, resulting in the formation of a yellow-colored 5-thio-2-nitrobenzoate complex. The absorbance of this complex was measured spectrophotometrically at 412 nm to determine AChE activity.

2.6. Influence of Temperature and pH on the AChE Activity

In order to determine the pH effect, the absorbance changes of free and immobilized AChE enzyme (immobilized enzyme was taken into different tubes at 1 mg each) activities were measured in the pH 5–11 range as specified in the method section (2.5. Enzyme Activity). In addition, to evaluate the temperature effect, the free and functionalized MNPs immobilized AChE enzyme were incubated at various temperatures between 20 and 80 °C for 2 h, and their final activities were measured.

2.7. Enzyme Kinetics and Stability Study

Fe₃O₄@TEOS/APTES/GA/AChE was added to the substrate solution (acetylthiocholine iodide; 0.02–0.1 μM) at different concentrations to evaluate the effect of substrate concentration on immobilized and free enzyme activity. Kinetic parameters were evaluated using tacrine, a competitive inhibitor of the enzyme, in the range of 10–60 μM. Then, *K_m* and *V_{max}* kinetic parameters were calculated from the Lineweaver-Burk calibration graph. A storage stability study was conducted to assess the longevity of immobilized enzymes at +4 °C over a 75-day period. Enzyme activity was measured at 15-day intervals and compared to the initial activity levels. To assess reusability, functionalized MNPs with AChE immobilized on the surface were washed with buffer solution after each reaction cycle and subsequently reintroduced into a fresh solution. The reaction cycles were conducted under controlled conditions (pH = 8.0, at room temperature), and enzyme activity was analyzed accordingly.

2.8. Statistical Studies

Data analysis and graphical visualizations were conducted using GraphPad Prism version 8 for Windows (GraphPad Software, La Jolla, California, USA), a software widely recognized for its robust statistical capabilities and user-friendly interface. Analyses were performed in 3 replicates, and results are expressed as means ± standard error of the mean.

3. Results and Discussion

Immobilized and stabilized enzymes are widely utilized in various biotechnological applications, as their stability is crucial for transportation and storage. Immobilization enhances both the stability and reusability of enzymes, thereby providing significant economic advantages.^[27] In recent years, extensive research has been conducted on enzyme immobilization strategies. Among these, covalent immobilization offers stronger bonding between the enzyme and the carrier compared to adsorption or ionic immobilization. As a result, enzyme leakage from the carrier does not occur even in the presence of a substrate or a highly ionic solution. Furthermore, due to the firm attachment of the enzyme to the carrier, the immobilized enzyme can efficiently interact with the substrate.^[28] However, covalent immobilization may alter the enzyme's active site and conformational structure, leading to a reduction in enzymatic activity. To mitigate this loss, it is essential to anchor the enzyme to the carrier via functional groups that do not participate in catalytic activity within the active site.^[28,29]

In this study, functionalized Fe₃O₄ magnetic nanoparticles (MNPs) were synthesized. The AChE, an important enzyme in the hydrolysis of organophosphorus pesticides widely used in agriculture, was immobilized on these functionalized MNPs with a cross-linker GA. Then, the kinetic parameters and stability (storage and operational stability) studies of this immobilized enzyme were determined by the spectrophotometric method (Scheme 1). The immobilized enzyme (Fe₃O₄@SiO₂-NH₂/GA/AChE) was characterized by SEM-EDX and FTIR.

3.1. Characterization and Immobilization of AChE on Functionalized MNPs

The GA is one of the most widely used bifunctional cross-linking agents. The covalent nature of the interaction can exhibit minimal leakage of enzymes and increased operational stability (even under harsh conditions).^[30] In literature studies with different matrices and methods, it has been reported that the concentration of the cross-linker glutaraldehyde, the amount of enzyme, and binding times have an impact on immobilization efficiency.^[22] In this study, the appropriate GA concentration optimized in the literature was studied. In order to evaluate the immobilization efficiency, the % immobilization rate was calculated after the AChE enzyme was bound to the support material (functionalized MNPs). This enzyme was 68% immobilized on functionalized MNPs. As a result of the spectroscopic analysis, the absence of protein in the support material filtrates after immobilization (after washing 3 times) and the absence of any decrease in the activities of the immobilized enzymes are thought to indicate that immobilization was achieved with minimal leakage by cross-linking.

Scanning electron microscopy (SEM) was employed to investigate the morphology and surface characteristics of the synthesized materials. The SEM analysis was performed for MNPs, amine-functionalized MNPs (Fe₃O₄@SiO₂-NH₂), and the immobilized AChE system. As illustrated in Figure 1, the Fe₃O₄ MNPs exhibited a predominantly spherical morphology with smooth surfaces and relatively uniform distribution, with an average particle size of approximately 10 nm. Upon surface modification with amine groups, the nanoparticles showed increased aggregation, with more irregular and complex surface features, indicating successful functionalization.

The SEM-EDX analysis was conducted to examine the morphological and elemental changes on the functionalized MNPs surface before and after AChE immobilization. As shown in Figure 2a, the functionalized MNPs exhibited a relatively smooth surface prior to immobilization. However, following AChE attachment, an increase in surface roughness was observed (Figure 2b), indicating successful enzyme immobilization. These findings confirm the effective cross-linking (with GA) of AChE onto the functionalized MNPs surface.

The EDX spectra were obtained for Fe₃O₄@SiO₂-NH₂/GA MNPs and Fe₃O₄@SiO₂-NH₂/GA/AChE MNPs. Peaks associated with Fe and O were obtained in the EDX spectra. Approximately the same EDX spectrum for Fe was obtained for both functionalized MNPs. However, S-related peaks were also seen in

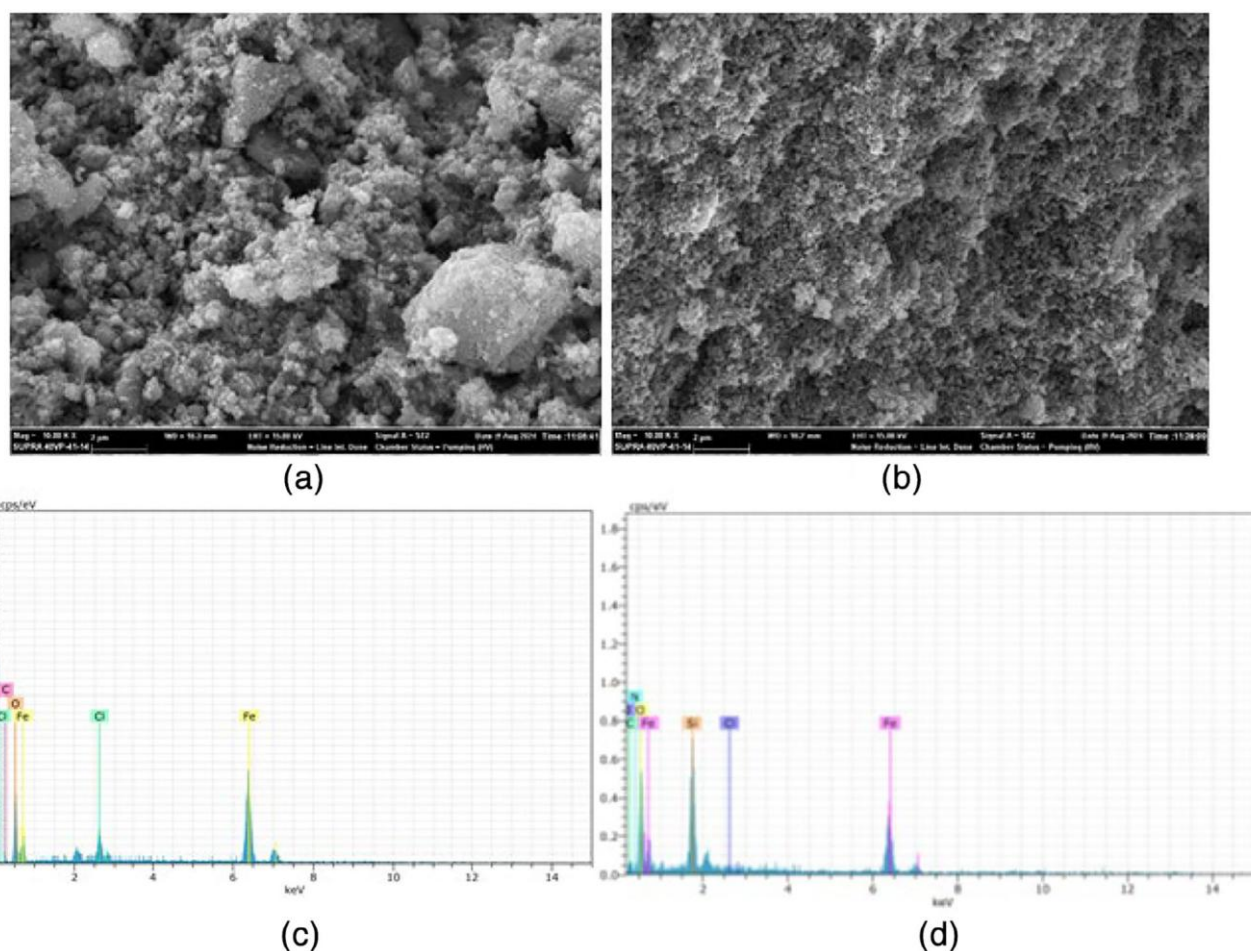
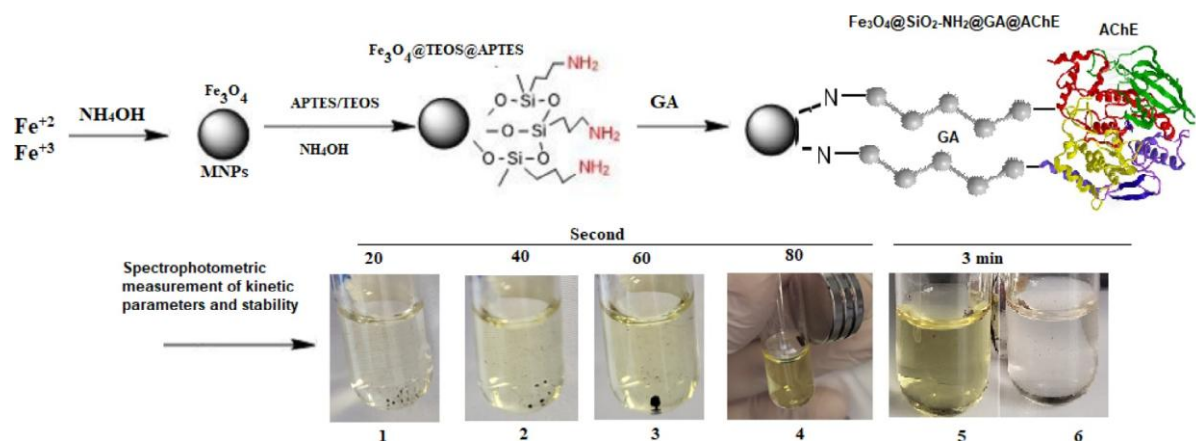


Figure 1. SEM-EDX analysis of a), c) Fe_3O_4 and b), d) Fe_3O_4 @ SiO_2 - NH_2 in functionalization of MNPs.

the spectrum of those with AChE bound to the surface of the MNPs (Figure 2d). The observed sulfur-related peaks may be attributed to the presence of sulfur-containing amino acids, such as methionine and cysteine, in the structure of AChE. This observation supports the successful immobilization of AChE on the

surface of the functionalized MNPs. The spectrum peaks showed a high Fe content, which may indicate good magnetic properties. In the experimental studies (Scheme 1), it is already predicted that the effective separation of MNPs with a magnet will be due to the magnetic property.

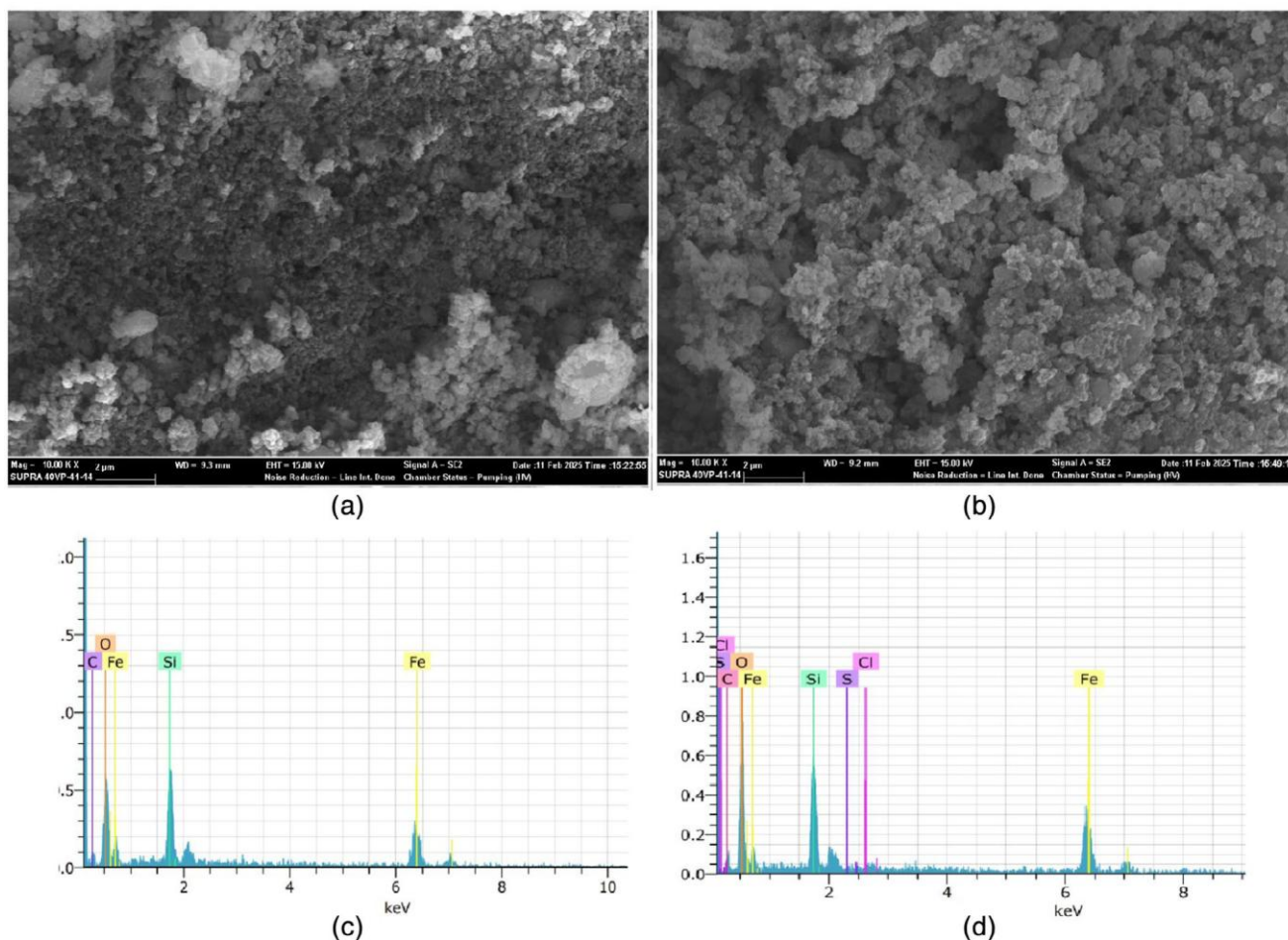


Figure 2. SEM-EDX analysis of a), c) Fe₃O₄@SiO₂-NH₂/GA and b), d) Fe₃O₄@SiO₂-NH₂/GA/AChE after immobilization.

FT-IR spectra of functionalized MNPs before and after immobilization are presented in the supplementary material (Figures S1 and S2). In the FTIR measurement results, transmission spectra were obtained between 400 and 4000 cm⁻¹. Before immobilization, 1055.63 cm⁻¹ (Si—O—Si stretching) and 2938.86 cm⁻¹ (C—H stretching) peaks indicate the presence of TEOS. The peaks at 557.92 and 795.69 cm⁻¹ may be related to Si—C stretching from APTES. Moreover, the presence of peaks at 1447.49, 1647.23, and 2166.05 cm⁻¹ indicates the presence of Si—O—Si, C=O, and amine groups, confirming the presence of TEOS, APTES, and GA. After immobilization, in the AChE spectrum, 1802.06 cm⁻¹ and 1587.70 cm⁻¹ show the stretching vibration of C=O and bending vibration of N—H, respectively. Moreover, the absorption peak at 560.25 cm⁻¹ corresponds to the stretching vibration of Fe—O bonds in Fe₃O₄. This indicates that AChE is fixed to the surface of MNP/TEOS/APTES.

The FTIR analysis of the synthesized and functionalized nanoparticles, as well as the immobilized AChE, supports the successful stepwise modification and enzyme binding processes. In a previous study, the characteristic peaks observed at 3340 and 1618 cm⁻¹ were attributed to the O—H stretching and deformation vibrations of Fe₃O₄ MNPs, respectively. Additionally, the absorption bands at 2920 cm⁻¹ and 2851 cm⁻¹ were associated with —CH₂ and C—H stretching vibrations in amine-

functionalized MNPs (MNPs-NH₂). Following immobilization, the shifts in the C=O stretching and N—H bending vibrations, appearing at 1660 and 1537 cm⁻¹, respectively, were interpreted as evidence of successful AChE binding onto the nanoparticle surface through covalent interactions.^[31] Our findings are consistent with these results. In the FTIR spectra obtained in this study, similar characteristic peaks were observed across the relevant modification stages. The spectral shifts associated with the immobilized AChE suggest conformational changes due to enzyme binding, and they align well with the aforementioned literature.

In a study, a new matrix was developed using alginate and carrageenan from different sources for the immobilization of AChE. The optimum temperature for immobilized AChE and free AChE was found to be about 30 °C. At 40 °C, the free and immobilized enzymes retained their activities up to about 20% and 49%, respectively.^[32] In this study, the optimum pH was 25 for free enzyme and 40 °C for immobilized enzyme. The thermal stability of the immobilized enzyme between 40–60 °C was higher than that of the free enzyme and the aforementioned study (Figure 3).

Enzyme activity was determined between pH 5 and 11 for both free and immobilized AChE activity. Figure 4 shows that the free enzyme reached the highest relative activity at pH

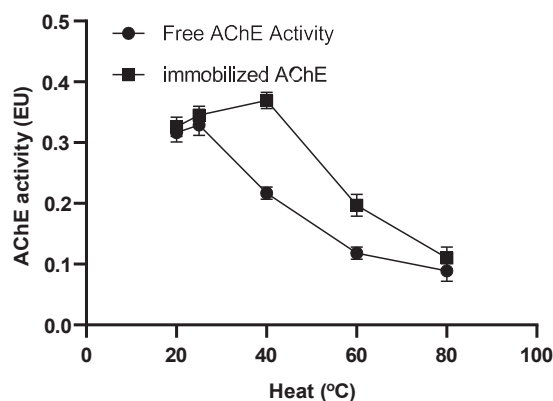


Figure 3. The thermal stability for free and immobilized AChE onto $\text{Fe}_3\text{O}_4/\text{SiO}_2\text{-NH}_2/\text{GA}$.

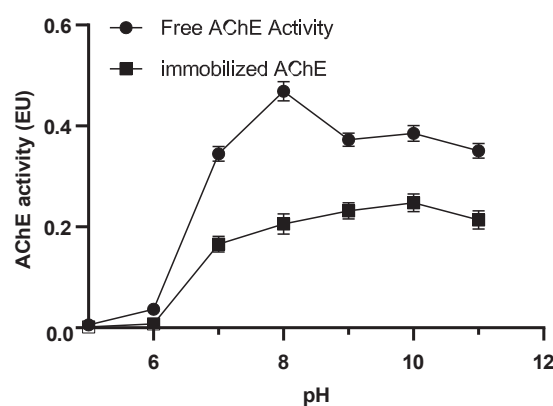


Figure 4. The optimal pH profile for free and immobilized AChE onto $\text{Fe}_3\text{O}_4/\text{SiO}_2\text{-NH}_2/\text{GA}$.

8, and the immobilized enzyme reached the highest relative activity at pH 10. It was observed that the pH-dependent enzyme activity increases, and the activity decreases after pH 8 for the free enzyme and after pH 10 for the immobilized enzyme. In a study, the optimum pH of the free enzyme and AChE enzyme immobilized on magnetic bio-nano composites ($\text{Fe}_3\text{O}_4/\text{Cht}/\text{Au}$) was found to be 8.^[21] In a previous study, a slight shift in the optimum pH of cross-linked enzymes was reported, which was attributed to interactions between the GA and surface or residual charge groups during the cross-linking

Table 1. Kinetic parameters (K_m and V_{max}) for free and immobilized AChE.			
Enzyme samples	K_m (μM)	V_{max} (EU mL^{-1})	K_i (μM)
Free AChE	0.163 ± 0.01	0.965 ± 0.07	12.10 ± 1.02
Immobilized AChE	0.446 ± 0.03	0.659 ± 0.05	21.64 ± 1.84

process.^[33] In line with this, the observed change in the optimum pH in our study may similarly result from such interactions occurring during enzyme immobilization. These findings suggest that glutaraldehyde-mediated cross-linking can influence the microenvironment of the enzyme, thereby affecting its catalytic behavior under different pH conditions.

In the study, the pH stability of free and immobilized AChE was determined in the pH 6–10 range for 1–2 days. As shown in Figure 5, free and immobilized enzymes were more stable in the pH 8–10 range. While the immobilized enzyme is more stable at pH 10, this value for the free enzyme is in the pH 8–10 range. When the results are evaluated, it is thought that this change may be due to the pH-dependent change in the three-dimensional conformational structure and immobilization. The fact that the optimum and stable pH varies in the range of 8–10 depending on the AChE source and support material in literature studies^[21,32] supports these findings.

3.2. Kinetic Properties

In a study, the AChE was immobilized on a newly synthesized support material for the free enzyme. When the kinetic parameters were evaluated, K_m and V_{max} values for free enzyme were found to be 6.35 and 50 mM min^{-1} , respectively, while these values for immobilized enzymes were determined as 8.68, 12.7, 39.7, and 52.9 mM min^{-1} , respectively.^[32] A previous study on the immobilization of AChE onto nanofibrous PVA/BSA membranes reported an approximately 1.7-fold increase in the K_m value of the immobilized enzyme compared to its free form.^[34] In the present study, the kinetic parameters of both free and immobilized AChE were determined using ATChI as the substrate at varying concentrations. The obtained results were compared with those of the free enzyme (Table 1). The K_m and V_{max} values for the immobilized AChE were found to be 0.446 mM and 0.659 EU mL^{-1} ,

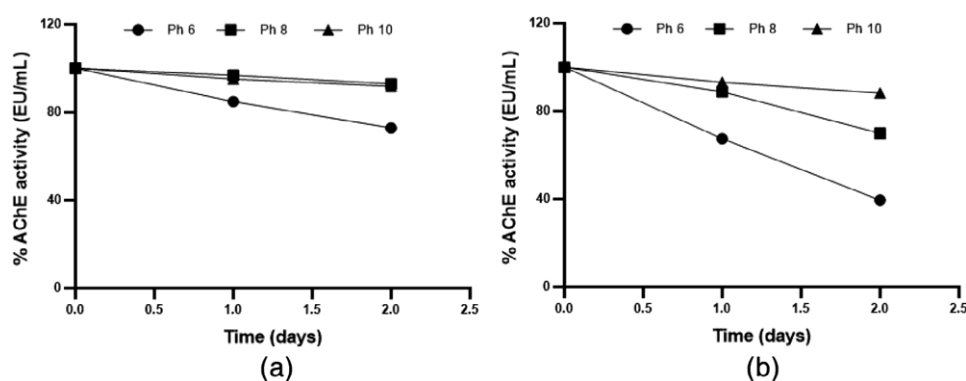


Figure 5. The pH stability profile for free a) and immobilized b) AChE ($\text{Fe}_3\text{O}_4/\text{SiO}_2\text{-NH}_2/\text{GA}/\text{AChE}$).

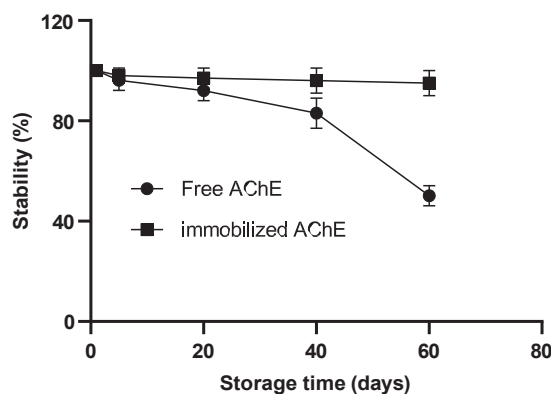


Figure 6. Storage stability of free and immobilized AChE in tris/HCl buffer (20 mM, pH 7.5).

respectively, while the corresponding values for the free AChE were 0.163 mM and 0.965 EU mL⁻¹. When the K_i value, which is an indicator of the binding affinity of tacrine, a known inhibitor of the AChE enzyme, to the enzyme, is examined, the binding affinity of tacrine to the immobilized enzyme is lower than that of the free enzyme with a lower K_i value.

Previous studies have indicated that the substrate binding efficiency to the active site of immobilized enzymes tends to decrease due to various factors.^[35] The observed increase in K_m and decrease in V_{max} values suggest that AChE immobilized on MNPs surfaces exhibits a reduced affinity for its substrate. This reduction may result from restricted enzyme mobility upon attachment to the MNPs surface, leading to potential alterations in its three-dimensional conformation. Additionally, steric hindrances arising from the immobilization process could further contribute to the diminished substrate accessibility and enzymatic activity. The binding of AChE to the surface of functionalized MNPs via glutaraldehyde may limit the enzyme's flexibility and introduce steric hindrance, thereby affecting the orientation and positioning of the substrate relative to the catalytic site. Moreover, the immobilization matrix may create a microenvironment that differs from the bulk solution in terms of pH, polarity, or charge distribution, further influencing enzymatic activity. These factors together can account for the observed kinetic behavior.

3.3. Stability and Reusability

A study on the immobilization of the AChE onto nanofibrous PVA/BSA membranes demonstrated that the immobilized enzyme retained 90% of its catalytic activity after 100 days of storage in phosphate buffer solution at 4 °C. This finding highlights the enhanced stability of AChE upon immobilization.^[34] In another study, the AChE enzyme was immobilized on a bio-nanocomposite surface. After 30 days, free AChE lost less than 50% of its catalytic activity, while immobilized AChE retained 82% of its catalytic activity.^[21] In this study, free and immobilized AChE (5.32×10^{-3} EU) were stored at 4 °C for a period of 60 days to assess their stability. As illustrated in Figure 6, immobilized AChE demonstrated significantly higher stability compared to its

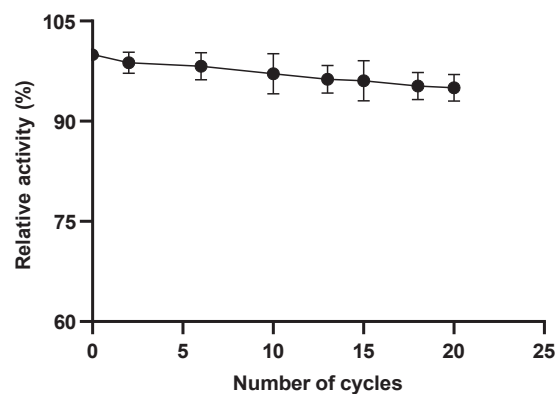


Figure 7. The operational stability (reusability) of immobilized AChE ($\text{Fe}_3\text{O}_4@\text{SiO}_2\text{-NH}_2/\text{GA}/\text{AChE}$).

free counterpart. While free AChE retained approximately 50.4% of its initial activity after 60 days, immobilized AChE preserved 95.2% of its activity even after 60 days. This enhanced stability is likely attributed to the cross-linking between the enzyme and the support material, which reinforces the conformational integrity of the immobilized enzyme. These findings suggest that immobilization contributes to an extended shelf life compared to the free enzyme.

In a study, the AChE immobilized on PANCHI-B membranes exhibited less than a 50% loss in enzyme activity after 10 reaction cycles.^[34] Similarly, when immobilized on another carrier material, AChE retained approximately 50% of its initial activity even after 10 cycles of reuse.^[36] These results demonstrate the enhanced reusability and stability of immobilized AChE on different support materials. In our study, the reusability of the immobilized AChE enzyme was evaluated over 20 consecutive cycles, as illustrated in Figure 7. The results indicated that the immobilized AChE retained over 95% of its initial activity, demonstrating its high stability and durability.

In contrast, the immobilized AChE in the present study exhibited exceptional reusability performance. As shown in Figure 7, the enzyme maintained more than 95% of its initial activity even after 20 consecutive reaction cycles. This superior retention of activity suggests that the attachment of AChE onto functionalized $\text{Fe}_3\text{O}_4@\text{SiO}_2\text{-NH}_2$ nanoparticles via glutaraldehyde provides a highly stable immobilization matrix. The improved durability may be attributed to the robustness of the magnetic support, the strength of the covalent linkage, and the protective microenvironment created by the functional groups on the nanoparticle surface. These findings highlight the potential of this immobilization strategy for applications requiring repeated enzymatic activity over extended operational periods.

4. Conclusions

This study presents an approach for the immobilization of the AChE enzyme onto functionalized MNPs using glutaraldehyde as a cross-linker, providing enhanced stability and reusability. The synthesized and functionalized MNPs were comprehensively characterized by FTIR, SEM, and EDX techniques. Compared to

the free enzyme, the immobilized AChE exhibited significantly improved thermal and pH stability, prolonged storage and operational lifespan, and favorable kinetic properties. The enhanced catalytic performance under optimized conditions demonstrates the potential of this immobilization strategy for applications in biosensing, biocatalysis, and environmental monitoring. The integration of magnetic separability with enzyme immobilization offers a reusable, efficient, and scalable system, which distinguishes this work from existing literature.

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Conflict of Interests

The authors declare no conflict of interest.

Author Contributions

S.B.: Methodology; resources and validation; writing—original draft. **M.I.:** Conceptualization and project administration; investigation; writing—original draft; software; resources and validation.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supporting Information

The FTR spectra of functionalized MNPs in enzyme-bound and unbound states are presented as supplementary material.

Keywords: Acetylcholinesterase immobilization • Enzyme kinetic • Enzyme stability • Functionalization • Magnetic nanoparticles

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