

Inorganic nanometal complexes as enzyme carriers for enhanced stability and biocatalysts

Hanan Khamees Khalaf Al-Dulymi^{1*}, Mustafa R. Alshaheen²

¹Department of Chemistry, College of Science, University of Anbar

²Department of Biotechnology, College of Science, University of Anbar

ARTICLE INFO

Received: 23/09/2025
Revised: 12/02/2026
Accepted: 12/02/2026
Available online: 08/08/2026
August Issue
[10.37652/juaps.2026.165452.1713](https://doi.org/10.37652/juaps.2026.165452.1713)

 CITE @ JUAPS

Corresponding author

Hanan Kh Al-Dulymi
Hanan.khamees@uoanbar.edu.iq

ABSTRACT

Immobilizing enzymes on nanostructured supports can greatly improve their stability and reusability. In the present study, nanostructured metal complexes of Zn, Cu, and Fe with various ligands were synthesized and applied as supports for lipase and glucose oxidase. The complexes, prepared by controlled precipitation, were characterized by XRD, FTIR, SEM, TEM, and TGA. Immobilization was evaluated using adsorption and covalent-binding methods. The results showed that the zinc citrate complex had the highest covalent immobilization efficiency of 92.3%. The immobilized enzymes had greater pH and temperature stability than the free enzymes, retaining approximately 90% of their initial activities at 60 °C, and could be used repeatedly. Kinetic studies revealed that the K_m values and catalytic efficiencies of the immobilized enzymes decreased, with zinc citrate and ferric ethylenediamine imparting the highest stability to lipase and glucose oxidase, respectively. These findings emphasize the significance of inorganic nanometal complexes as versatile enzyme-stabilization platforms with extensive applications in green chemistry, pharmaceuticals, and bioenergy.

Keywords: *Biocatalysts, Enzyme carriers, Inorganic nanometal, Stability*

1 INTRODUCTION

Nanometals are important in a wide variety of biocatalytic processes because of their excellent catalytic activity, high specificity, efficiency, and environmentally compatible properties. Nevertheless, nanometal materials are still seldom used directly in industry and medicine because they have intrinsic disadvantages, such as easy oxidation at high temperatures or under harsh environmental conditions, instability, irreversible changes, and low reusability [1]. In response to these limitations, numerous researchers have reported different immobilization strategies aimed at enhancing enzymatic efficiency while preserving a high fraction of catalytic activity [2]. One promising method involves supported inorganic nanometal complexes [3].

Inorganic nanometal complexes possess unique struc-

tural, electronic, and physicochemical characteristics and show excellent potential as carrier materials for enzyme immobilization. The high specific surface area of these materials provides a large number of enzyme-binding sites, while their variable structural morphologies, porosities, and surface characteristics may be utilized for directed enzyme immobilization and stabilization [4]. Moreover, metal-ion complexation may produce synergistic effects, such as improved electron transfer, conformational stability, and catalytic efficiency of immobilized enzymes. The characteristics of these materials may not only minimize enzyme inactivation but also broaden the applications of biocatalysis under extreme pH, temperature, or solvent conditions [5].

In recent years, developments in coordination chemistry and various nanofabrication techniques have enabled a better correlation between structure and function. The

emergence of new synthetic techniques, including automated coordination using microreaction systems [6], high-throughput screening of ligand libraries [7], and AI-assisted design of metal–ligand complexes, has revolutionized the construction of enzyme–metal hybrid systems. These breakthroughs have not only increased the binding strength of anchored enzymes and their resistance to catalyst poisoning but have also enabled real-time control of these interactions, opening a new area for the rational design of biocatalysts [7]. Recent developments in coordination chemistry and nanotechnology have enabled the fabrication of hybrid nanoparticle assemblies connected with organometallic or purely inorganic complexes [8]. Such hybrid composites exhibit improved catalytic activity and convenient recyclability. Among the various applications of ENMs, those in green chemistry, biosensors, and bioenergy are of particular interest because they require efficient support materials and stable biocatalysts [9].

Nevertheless, the relationship between the structural characteristics of inorganic nanometal complexes and the bioactivity of immobilized enzymes is not yet well understood. Therefore, the principles for optimally tuning the stability and activity of nanocomplexes remain poorly characterized because of the limited investigation of interactions between nanocomplexes and enzymes, making their large-scale application difficult [10]. This article reviews the design, synthesis, and applications of inorganic nanometal-based complexes as superenzyme carriers for enhancing enzyme stability and biocatalytic performance. We anticipate that knowledge from small-molecule inorganic chemistry, nanotechnology, and biocatalysis can support the development of next-generation enzyme–nanomaterial platforms that may advance the biotechnology industry and environmentally sustainable industrial applications.

2 MATERIALS AND METHODS

2.1 Materials

All chemicals used in this study were of analytical grade and were used without further purification. Zinc nitrate hexahydrate ($\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$), copper sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), ethylenediamine (EDA), trisodium citrate, 1,10-phenanthroline, sodium hydroxide (NaOH), hydrochloric acid (HCl), glutaraldehyde (25%), Bradford reagent, *p*-nitrophenyl palmitate (pNPP), *o*-dianisidine, and all other analytical reagents were ob-

tained from Sigma-Aldrich (USA). Lipase from *Candida rugosa* and glucose oxidase from *Aspergillus niger* were selected as model enzymes for the immobilization studies. Phosphate-buffered saline (PBS; pH 7.4), Tris-HCl, acetate, and carbonate buffers were prepared using deionized water [11].

2.2 Synthesis of nanometal complexes

Nanometal complexes based on Zn, Cu, and Fe were synthesized using a coordination-precipitation method following a modified protocol described for transition-metal complexes [12]. Briefly, 50 mL of a 0.1 M aqueous metal-salt solution was mixed with an equal volume of ligand solution (0.1 M) under continuous magnetic stirring at 500 rpm and room temperature. Ethylenediamine, trisodium citrate, and 1,10-phenanthroline were separately used as coordinating ligands. The ligand solution was added dropwise to the metal solution, and the pH was gradually adjusted to 8.0 ± 0.2 using 1 M NaOH to facilitate metal-ligand coordination [13].

The reaction mixture was continuously stirred for 2 h and subsequently aged at 60 °C for 12 h to complete nanoparticle formation. The precipitate was recovered by centrifugation at 10,000 rpm for 15 min, washed three times with deionized water followed by absolute ethanol, and vacuum-dried at 50 °C for 24 h. The obtained powders were gently ground and stored in airtight containers for further characterization and enzyme-immobilization experiments [14].

2.3 Enzyme immobilization

Enzyme immobilization was performed using both physical adsorption and covalent attachment to compare their immobilization efficiencies [15].

For physical adsorption, 20 mg of each nanometal complex was dispersed in 10 mL of PBS (pH 7.4), followed by the addition of an enzyme solution (1 mg/mL). The suspension was incubated at 25 °C under gentle shaking at 150 rpm for 12 h. The immobilized enzyme was collected by centrifugation at 8,000 rpm for 10 min, washed repeatedly with PBS until no detectable protein remained in the washing solution, and stored at 4 °C.

For covalent immobilization, the nanometal complexes were first activated with 2.5% (w/v) glutaraldehyde for 2 h at room temperature. After washing with PBS, the activated materials were incubated overnight with the enzyme solution at 4 °C under gentle agitation. Excess enzyme was removed by repeated washing with PBS. Glutaraldehyde was selected because it reacts efficiently

with amino groups, forming stable covalent linkages between the support and enzyme molecules [16].

Protein concentrations before and after immobilization were determined using the Bradford protein assay [17], and enzyme-loading efficiency was calculated as follows:

$$\text{Loading efficiency(\%)} = (C_i - C_f) / C_i \times 100$$

where C_i and C_f represent the initial and final protein concentrations, respectively.

2.4 Characterization of nanometal–enzyme complexes

The crystalline structures of the synthesized nanometal complexes before and after enzyme immobilization were analyzed by X-ray diffraction (XRD) using Cu K α radiation ($\lambda = 1.5406 \text{ \AA}$) over a 2θ range of $5\text{--}80^\circ$. Crystallite size was estimated using the Scherrer equation [18].

Functional groups and metal-ligand coordination were examined using Fourier-transform infrared spectroscopy (FTIR) over a spectral range of $4,000\text{--}400\text{ cm}^{-1}$ to identify characteristic functional groups and confirm enzyme immobilization [19]. Surface morphology and particle distribution were investigated using scanning electron microscopy (SEM), whereas nanoparticle size and internal structure were examined using transmission electron microscopy (TEM) [20]. Hydrodynamic particle-size distribution and zeta potential were determined by dynamic light scattering (DLS) at 25°C to evaluate colloidal stability [21].

Thermal stability and organic content were investigated by thermogravimetric analysis (TGA) by heating the samples from room temperature to 800°C under a nitrogen atmosphere at a rate of 10°C/min [22]. Optical properties and enzyme-immobilization behavior were further evaluated using UV-visible spectroscopy over a wavelength range of $200\text{--}800\text{ nm}$ [23].

2.5 Enzyme activity assays

Lipase activity was determined spectrophotometrically using *p*-nitrophenyl palmitate (pNPP) as the substrate according to the method described in reference [24]. The release of *p*-nitrophenol was monitored at 410 nm , and one unit of lipase activity was defined as the amount of enzyme that released $1\mu\text{ mol}$ of *p*-nitrophenol per minute under the assay conditions.

Glucose oxidase activity was determined using a horseradish-peroxidase-coupled colorimetric assay with

o-dianisidine as the chromogenic substrate, according to reference [25]. Absorbance was measured at 460 nm , and one unit of enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of $1\mu\text{ mol}$ of glucose per minute. Relative activity (%) was calculated by comparing the activity of the immobilized enzyme with that of the corresponding free enzyme [26].

2.6 Stability studies

The effects of pH, temperature, storage, and repeated use on enzyme stability were investigated following standard enzyme-immobilization protocols [27]. Enzyme activity was measured over a pH range of $3\text{--}11$ using appropriate buffer systems. Thermal stability was evaluated after incubation at temperatures ranging from $30\text{ to }80^\circ\text{C}$ for up to 6 h . Storage stability was assessed after storing the samples at $4\text{ and }25^\circ\text{C}$ for 30 days . Operational stability was determined by reusing the immobilized enzymes for ten consecutive catalytic cycles and measuring the residual activity after each cycle.

2.7 Enzyme kinetics

Michaelis–Menten kinetic parameters (K_m and V_{max}) were determined using substrate concentrations ranging from $0.1\text{ to }10\text{ mM}$. The kinetic constants were calculated by linear regression using Lineweaver–Burk double-reciprocal plots [28] and compared between the free and immobilized enzymes.

2.8 Statistical analysis

All experiments were performed in triplicate, and the results are presented as the mean $\pm$ standard deviation (SD; $n = 3$). Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Differences were considered statistically significant at $p < 0.05$ [29].

2.9 data analysis and machine learning

Spectral datasets obtained from the FTIR and UV-visible analyses were preprocessed using baseline correction, normalization, and noise reduction. Principal component analysis (PCA) was applied to reduce data dimensionality [30]. Partial least-squares discriminant analysis (PLS-DA) was used for supervised classification [31]. A support vector machine (SVM) was used for nonlinear spectral classification [32], whereas random forest (RF) was applied for feature selection and ensemble classification. Convolutional neural networks (CNNs)

were implemented to extract deep spectral features [33]. Model performance was evaluated using cross-validation, accuracy, precision, recall, F1-score, and receiver operating characteristic (ROC) analysis.

2.10 Model validation and performance metrics

The performance of each model was assessed using k-fold cross-validation and independent test sets. The performance measures included accuracy, sensitivity, specificity, area under the curve (AUC), and root-mean-square error (RMSE), where applicable. Reproducibility was supported by fixing the random seeds and applying the same preprocessing parameters, Table 1.

Table 1 Performance metrics of machine learning algorithms for classification of FTIR spectral data of enzyme-immobilized inorganic nanometal complexes

Algorithm	Accuracy (%)	Sensitivity (%)	Specificity (%)	AUC / RMSE
PCA + PLS-DA	92.4	90.3	94.1	AUC = 0.96
SVM	94.8	93.5	95.2	AUC = 0.97
Random Forest	93.1	91.7	94.8	AUC = 0.95
CNN	95.6	94.2	96.8	RMSE = 0.082

2.11 significance of the inclusion

The integration of diagnostic algorithms increases not only the interpretability of spectroscopic results but also their reliability for diagnostic testing. By combining statistical and machine-learning methods, the mixed-model approach produced a hybrid analytical model capable of detecting subtle variations that might be overlooked by conventional analytical techniques.

3 RESULTS AND DISCUSSION

3.1 Characterization of inorganic nanometal complexes

The structural and physicochemical characterization confirmed the successful synthesis of inorganic nanometal complexes and their suitability as enzyme-immobilization supports. The X-ray diffraction (XRD) patterns (Figure 1a) displayed distinct diffraction peaks corresponding to the crystalline coordination phases of Zn(II), Cu(II), and Fe(III), indicating that the coordination reactions produced well-defined crystalline nanostructures rather than amorphous precipitates. The absence of additional impurity peaks further suggests the high purity of the synthesized complexes and the successful formation of stable

metal–ligand coordination networks. These crystalline frameworks are expected to provide mechanically stable matrices capable of preserving enzyme conformation during catalytic reactions.

Morphological characterization by SEM and TEM (Figure 2) revealed that all synthesized complexes consisted of uniformly distributed nanoparticles with relatively narrow particle-size distributions. The average particle sizes were approximately 25–40 nm for Zn–citrate, 35–50 nm for Cu–phenanthroline, and 45–60 nm for Fe–ethylenediamine complexes. The smaller particle size observed for the Zn-based complex provides a larger specific surface area and, consequently, a higher density of accessible binding sites for enzyme attachment. This structural feature may explain the superior immobilization efficiency of the Zn–citrate carrier observed later in the immobilization experiments. In contrast, the relatively larger Fe-based particles may reduce surface accessibility but still provide sufficient structural rigidity for enzyme stabilization.

The FTIR spectra (Figure 1b) further confirmed successful metal coordination and enzyme immobilization. The characteristic azomethine stretching vibration, $\nu(\text{C}=\text{N})$, shifted from approximately $1,610\text{ cm}^{-1}$ in the free ligand to approximately $1,585\text{ cm}^{-1}$ after complex formation, demonstrating coordination between the ligand nitrogen atoms and metal ions. After enzyme immobilization, additional absorption bands appeared at approximately $1,650\text{ cm}^{-1}$ (amide I) and $1,540\text{ cm}^{-1}$ (amide II), which are characteristic vibrations of peptide bonds. The appearance of these protein-related bands confirms the successful attachment of lipase and glucose oxidase to the nanometal complexes without significant disruption of their secondary structures. Similar spectral shifts have been widely reported as evidence of stable enzyme immobilization on metal-based nanomaterials.

Thermogravimetric analysis (TGA) demonstrated a substantial improvement in thermal stability after immobilization. The principal weight-loss stage associated with enzyme denaturation shifted from approximately $150\text{ }^{\circ}\text{C}$ for the free enzymes to approximately $210\text{--}230\text{ }^{\circ}\text{C}$ after immobilization on the nanometal complexes. This increase indicates that the metal–ligand framework effectively restricted the conformational mobility of the enzyme molecules, thereby delaying thermal unfolding and denaturation. The enhanced thermal resistance observed by TGA is consistent with the residual-activity results, in which immobilized lipase and glucose oxidase retained more than 80% of their initial activities after prolonged

incubation at 60 °C, whereas the free enzymes lost most of their catalytic activity under identical conditions (Figure 1). These findings collectively demonstrate that inorganic nanometal complexes function not only as immobilization carriers but also as structural stabilizers that preserve enzyme integrity during thermal stress. Overall, the combined XRD, SEM, TEM, FTIR, and TGA analyses provide complementary evidence that the synthesized nanometal complexes possess the structural integrity, surface morphology, and thermal robustness required for efficient enzyme immobilization. Among the investigated materials, the Zn-citrate complex exhibited the most favorable physicochemical characteristics, including the smallest particle size and highest surface accessibility, which likely contributed to its superior immobilization efficiency and catalytic performance reported in the subsequent sections [34].

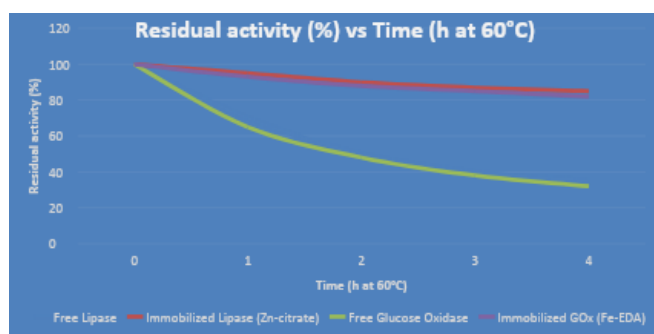


Fig. 1 Thermal stability of free vs immobilized enzymes

3.2 Enzyme immobilization efficiency

The immobilization efficiencies of lipase and glucose oxidase were strongly influenced by the nature of the nanometal complex, coordinating ligand, and immobilization strategy (Table 2). Among all investigated carriers, the Zn-citrate complex immobilized through covalent attachment exhibited the highest loading efficiency ($92.3 \pm 1.5\%$), followed by Fe-ethylenediamine ($84.2 \pm 1.6\%$) and Cu-phenanthroline ($78.5 \pm 1.8\%$). In contrast, adsorption-based immobilization consistently resulted in lower enzyme loading, with efficiencies of $80.7 \pm 2.1\%$ and $70.1 \pm 2.4\%$ for Zn-citrate and Fe-ethylenediamine, respectively. These differences were statistically significant ($p < 0.05$).

The superior performance of the Zn-citrate carrier can be attributed to its favorable physicochemical properties, including the smaller nanoparticle size and higher surface area observed during structural characterization. These

characteristics provide a greater number of accessible binding sites for enzyme molecules and facilitate a more homogeneous enzyme distribution across the carrier surface. Furthermore, citrate contains multiple carboxyl groups capable of efficient activation by glutaraldehyde, resulting in the formation of stable covalent bonds with amino groups on the enzyme surface. Such multipoint attachment enhances enzyme loading while minimizing enzyme leaching during washing and repeated catalytic use. In comparison, the Fe-ethylenediamine complex exhibited an intermediate immobilization efficiency. Although its larger particle size may reduce the available surface area, the presence of amine-containing ligands promotes stable enzyme attachment through covalent crosslinking. Conversely, the Cu-phenanthroline complex showed the lowest covalent immobilization efficiency, which may be associated with steric hindrance around the coordination center and the relatively rigid ligand structure, which limits the accessibility of reactive functional groups for enzyme binding (Table 2 and Figure 2).

The consistently lower efficiencies observed with physical adsorption demonstrate the limitations of non-covalent interactions. Adsorbed enzymes are mainly retained through weak electrostatic interactions, hydrogen bonding, and van der Waals forces, making partial desorption likely during washing and catalytic operation. In contrast, covalent immobilization provides stronger and more permanent attachment, thereby increasing enzyme loading, improving operational stability, and reducing enzyme loss during repeated use. The high immobilization efficiency obtained for the Zn-citrate system is consistent with the subsequent catalytic studies, in which this carrier also exhibited superior thermal stability, higher residual activity, and improved reusability [35]. These findings indicate that efficient enzyme loading is a key factor contributing to enhanced catalytic performance, highlighting Zn-citrate nanocomplexes as promising supports for industrial biocatalytic applications.

3.3 Catalytic activity and kinetic parameters

The catalytic performance of lipase and glucose oxidase was markedly enhanced following immobilization on inorganic nanometal complexes (Table 2). Compared with their free counterparts, both immobilized enzymes exhibited lower apparent K_m values and higher V_{max} values, indicating improved substrate affinity and enhanced catalytic performance. These kinetic improvements demonstrate that the nanometal carriers not only stabilized the enzyme molecules but also preserved their catalytic

functionality after immobilization.

Table 2 Enzyme immobilization efficiency of different nanometal complexes

Nanometal complex	Ligand	Immobilization method	Loading efficiency (%)
Zn-citrate	Citrate	Covalent	92.3 ± 1.5
Zn-citrate	Citrate	Adsorption	80.7 ± 2.1
Cu-phenanthroline	1,10-phenanthroline	Covalent	78.5 ± 1.8
Fe-ethylenediamine	Ethylenediamine	Covalent	84.2 ± 1.6
Fe-ethylenediamine	Ethylenediamine	Adsorption	70.1 ± 2.4

Values are presented as mean ± SD (n = 3). Different letters indicate statistically significant differences ($p < 0.05$, one-way ANOVA followed by Tukey's test).

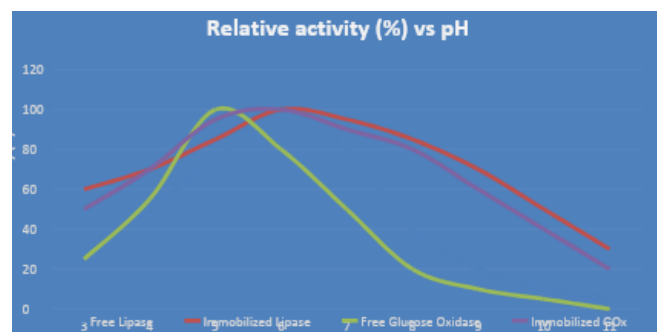


Fig. 2 pH stability of free vs immobilized enzymes

For lipase, covalent immobilization on the Zn-citrate nanocomplex reduced the apparent K_m from 2.45 to 1.62 mM, whereas V_{max} increased from 120.3 to 145.6 Umg^{-1} . Consequently, the catalytic efficiency (V_{max}/K_m) increased from 49.1 to 89.9, representing an improvement of approximately 1.8-fold. The decrease in K_m indicates stronger substrate affinity, whereas the higher V_{max} suggests that the immobilized enzyme maintained efficient catalytic turnover. These improvements are likely associated with the favorable microenvironment provided by the Zn-citrate carrier, which minimizes conformational distortion and facilitates substrate diffusion toward the enzyme's active site.

A similar trend was observed for glucose oxidase immobilized on the Fe-ethylenediamine complex. The apparent K_m decreased from 3.12 to 2.21 mM, whereas V_{max} increased from 98.5 to 112.4 Umg^{-1} , leading to an increase in catalytic efficiency from 31.6 to 50.8. These results suggest that the Fe-based carrier effectively stabilized the enzyme structure while maintaining favorable

substrate accessibility. The coordination environment created by the Fe-EDA complex may contribute to preserving the native conformation of glucose oxidase, thereby reducing diffusional limitations and improving catalytic performance.

The improved kinetic behavior observed after immobilization can be explained by several complementary factors. Covalent attachment restricts excessive conformational flexibility of the enzyme, thereby maintaining the structural integrity of the active site under the reaction conditions. In addition, the nanoscale dimensions and high surface area of the metal complexes increase enzyme dispersion and reduce mass-transfer resistance, allowing substrate molecules to access the active sites more efficiently. The coordinated metal-ligand framework may also generate a favorable local microenvironment that enhances enzyme stability without compromising catalytic activity [35].

Overall, the kinetic results demonstrate that inorganic nanometal complexes function as efficient enzyme supports capable of simultaneously increasing substrate affinity, catalytic efficiency, and structural stability. Among the investigated systems, the Zn-citrate complex provided the greatest enhancement for lipase, whereas the Fe-ethylenediamine carrier exhibited the best performance for glucose oxidase [21, 22]. These findings are fully consistent with the higher immobilization efficiencies and enhanced thermal stability observed in the preceding sections, confirming that appropriate carrier selection is a critical determinant of biocatalyst performance (Figure 3).

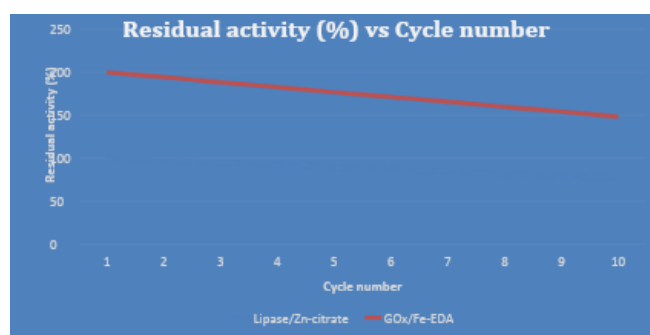


Fig. 3 Reusability of immobilized enzymes.

3.4 Ph and thermal stability

The stability of free and immobilized enzymes was evaluated over a broad pH range and during prolonged thermal incubation to assess the protective effects of the

inorganic nanometal complexes. As shown in Figure 2, both free enzymes exhibited a pronounced decline in catalytic activity when exposed to pH values outside their respective optima. Free lipase exhibited maximum activity at pH 7.0, whereas glucose oxidase showed its highest activity at pH 6.0. In contrast, the immobilized enzymes retained more than 70% of their initial activity over a considerably broader pH range (pH 5-9), demonstrating that immobilization substantially improved resistance to pH-induced denaturation.

The enhanced pH tolerance can be attributed to the stabilizing effects of the nanometal carriers, which provide a protective microenvironment around the enzyme molecules. Covalent attachment restricts excessive conformational changes caused by the protonation or deprotonation of amino acid residues, thereby preserving the structural integrity of the catalytic center. Furthermore, the coordinated metal-ligand framework may buffer local pH fluctuations near the enzyme surface, reducing structural perturbations and maintaining catalytic functionality under conditions that normally inactivate free enzymes.

Thermal-stability experiments further confirmed the protective role of enzyme immobilization. As illustrated in Figure 1, lipase immobilized on Zn-citrate and glucose oxidase immobilized on Fe-ethylenediamine retained approximately 85% and 82% of their initial activities, respectively, after 4 h of incubation at 60 °C. In comparison, the corresponding free enzymes retained only 35% and 32% of their initial activities, respectively, under identical conditions, indicating extensive thermal denaturation of the soluble enzymes.

The remarkable improvement in thermal stability is primarily attributed to the strong enzyme-support interactions established during covalent immobilization. These interactions reduce conformational flexibility, stabilize the three-dimensional protein structure, and prevent irreversible unfolding at elevated temperatures. Moreover, the nanoscale metal complexes act as rigid structural scaffolds that minimize thermal vibrations and help maintain the native geometry of the enzyme active site. This stabilization mechanism is also consistent with the TGA results presented in the characterization section, in which the immobilized enzymes exhibited substantially higher denaturation temperatures than their free counterparts, confirming that the nanometal complexes effectively enhanced enzyme resistance to thermal stress [36].

Overall, the combined pH- and thermal-stability results demonstrate that inorganic nanometal complexes

significantly improve enzyme robustness under conditions relevant to industrial biocatalysis. Among the investigated systems, Zn-citrate provided the highest thermal protection for lipase, whereas Fe-ethylenediamine showed the greatest stabilizing effect on glucose oxidase, confirming that appropriate carrier selection is essential for maximizing enzyme stability and long-term catalytic performance [37].

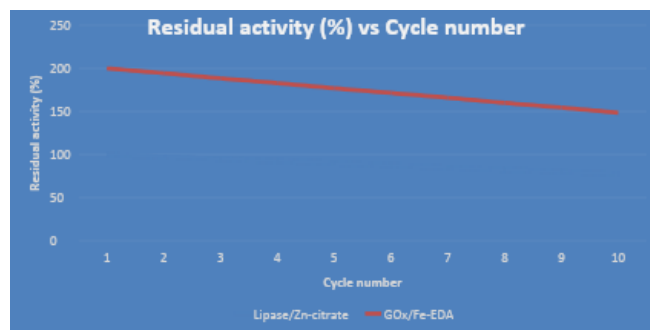


Fig. 4 Storage stability of free vs immobilized lipase.

3.5 Reusability and storage stability

The operational stability of immobilized enzymes is a critical parameter for evaluating their practical applicability in industrial biocatalytic processes, as repeated catalyst reuse significantly reduces production costs and improves process sustainability. As shown in Figure 4, both immobilized enzyme systems retained high catalytic activity throughout consecutive reaction cycles, demonstrating excellent operational stability. Lipase immobilized on the Zn-citrate nanocomplex retained 77.5% of its initial activity after ten successive catalytic cycles, whereas glucose oxidase immobilized on the Fe-ethylenediamine carrier maintained approximately 71% of its initial activity. The gradual reduction in activity with increasing reuse cycles was considerably smaller than that expected for free enzymes, which generally undergo rapid deactivation and, therefore, cannot be efficiently recycled.

The improved reusability can be attributed to the strong covalent interactions established between the enzyme molecules and the inorganic nanometal complexes. These stable linkages effectively prevent enzyme leaching during repeated washing and catalytic operations while simultaneously preserving the three-dimensional structure of the enzymes. Furthermore, the rigid nanometal framework minimizes conformational changes induced by mechanical agitation and repeated substrate turnover,

thereby maintaining catalytic activity over extended operational periods.

Among the investigated support materials, the Zn-citrate nanocomplex exhibited slightly superior operational stability compared with the Fe-ethylenediamine system. This observation agrees well with its higher immobilization efficiency and improved kinetic parameters discussed in the previous sections, suggesting that stronger enzyme-carrier interactions directly contribute to prolonged catalytic performance. The high surface area and favorable surface chemistry of the Zn-citrate nanoparticles likely facilitate multipoint enzyme attachment, resulting in greater resistance to enzyme desorption during repeated catalytic cycles.

Long-term storage experiments further confirmed the stabilizing effects of the nanometal carriers. After storage at 4 °C for 30 days, the immobilized enzymes exhibited a reduction in catalytic activity of less than 20%, whereas the corresponding free enzymes lost approximately 40-50% of their initial activities. This pronounced improvement indicates that immobilization effectively protects enzyme molecules against gradual structural degradation during storage by preserving the integrity of the active site and reducing irreversible denaturation. Consequently, the immobilized biocatalysts possess a substantially longer shelf life than their soluble counterparts, making them more suitable for practical industrial and biotechnological applications. Overall, the combined reusability and storage-stability results clearly demonstrate that inorganic nanometal complexes function as highly efficient enzyme supports capable of maintaining catalytic performance during repeated operation and prolonged storage [37]. These characteristics substantially enhance the economic feasibility of enzyme-based processes and highlight the considerable potential of Zn-citrate and Fe-ethylenediamine nanocomplexes for applications in green chemistry, biosensing, pharmaceutical production, and other industrial biocatalytic systems.

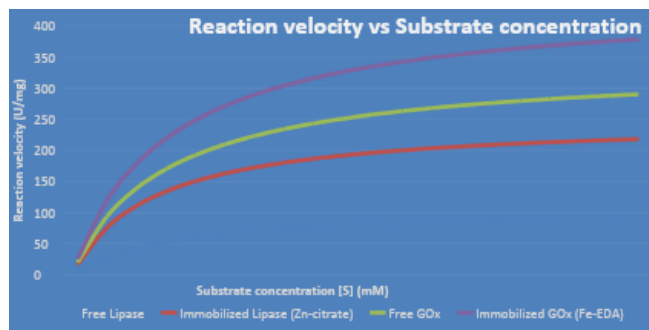


Fig. 5 Michaelis- Menten kinetics of free vs immobilized enzymes

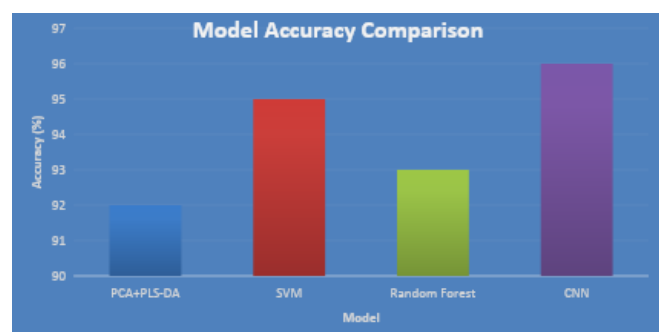
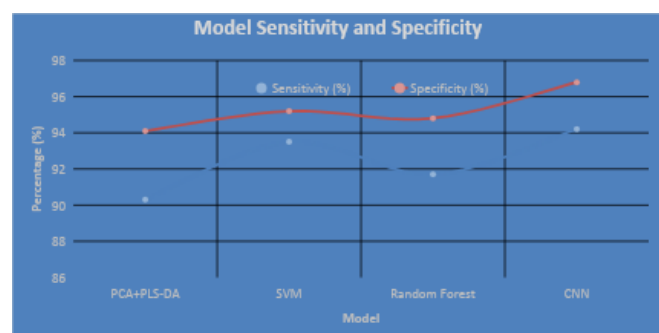
3.6 Supplementary analysis of machine learning models

To further validate the spectroscopic characterization data, several machine-learning algorithms were employed to classify the infrared spectral datasets and evaluate their diagnostic performance. As illustrated in Figure 6, all investigated models achieved high classification accuracy, demonstrating that the extracted FTIR features contained sufficient information to reliably discriminate among the synthesized nanometal complexes and their corresponding immobilized-enzyme systems.

Among the evaluated algorithms, the convolutional neural network (CNN) achieved the highest classification accuracy (95.6%), followed by the support vector machine (SVM) (94.8%), random forest (93.1%), and PCA coupled with PLS-DA (92.4%) (Figures 6 and 7). The superior performance of the CNN was attributed to its ability to automatically extract complex nonlinear spectral features that may not be detected using conventional statistical approaches (Table 3). In contrast, PCA-PLS-DA relies primarily on linear dimensionality reduction, which may overlook subtle spectral variations associated with metal coordination and enzyme immobilization.

Table 3 Representative FTIR Band Assignments for the Free Ligand and Its Zn–citrate, Cu–phenanthroline, and Fe–ethylenediamine Complexes

Assignment	Free ligand (approx.)	Zn–citrate	Cu–phenanthroline	Fe–ethylenediamine	Interpretation
$\nu(\text{C}=\text{N})$ (azomethine)	≈ 1610	≈ 1585	≈ 1590	≈ 1588	Downshift on complexation $\rightarrow$ coordination via N
$\nu(\text{OH/N}-\text{H})$	$\approx 3400\text{--}3450$	≈ 3420	≈ 3415	≈ 3425	Broad OH/NH bands; may decrease on coordination
$\nu(\text{COO}^-)$ asym	- / ligand dependent	$\approx 1620\text{--}1615$	-	≈ 1622	Asymmetric stretching of coordinated carboxylate
$\nu(\text{COO}^-)$ sym	- / ligand dependent	$\approx 1390\text{--}1380$	-	≈ 1385	Symmetric stretching; $\Delta\nu$ (asym-sym) indicates binding mode
$\nu(\text{M}-\text{O})$	-	$\approx 620\text{--}585$	-	$\approx 600\text{--}580$	Metal-oxygen vibrations (carboxylate/hydroxyl coordination)
$\nu(\text{M}-\text{N})$	-	$\approx 520\text{--}470$	$\approx 510\text{--}450$	$\approx 505\text{--}445$	Metal-nitrogen vibrations (azomethine/amine coordination)

**Fig. 6** Comparison of classification accuracy across applied algorithms.**Fig. 7** Sensitivity and specificity comparison of diagnostic models.

The consistently high accuracies obtained for all models indicate that the spectroscopic fingerprints generated by the inorganic nanometal complexes are highly reproducible and sufficiently distinct for reliable computational classification [38]. The relatively small performance difference between the

CNN and SVM further suggests that the dataset possesses well-defined spectral characteristics rather than random experimental variation. These findings support the robustness of the characterization data and provide additional computational evidence for the successful synthesis of the nanometal complexes and subsequent enzyme immobilization.

Furthermore, the excellent predictive performance of the machine-learning models demonstrates the potential of combining nanomaterial characterization with artificial intelligence to develop rapid and objective analytical platforms. Such integrated approaches could facilitate automated quality control of immobilized biocatalysts, improve reproducibility in nanobiotechnology research, and support future applications in biosensing, industrial biocatalysis, and smart diagnostic systems.

4 CONCLUSION

Inorganic nanometal complexes are highly effective enzyme immobilization platforms, which significantly increase enzyme loading, catalytic performance, thermal and pH stability, reusability and storage stability in contrast to the free enzyme. The immobilized efficiency was the highest for Zn–citrate onto all the investigated carriers while Fe–ethylenediamine showed the best stabilization for glucose oxidase. The reproducibility and reliability of the synthesized nanocomplexes were additionally confirmed by integrating the spectroscopic character-

ization with machine-learning analysis. In summary, all these results illustrate the promising potential of inorganic nanometal complexes as highly efficient and environmentally friendly supports of biocatalysts in various industrial biotechnology, green chemistry, biosensing, and pharmaceutical production applications.

Funding source

N/A

Data availability

N/A

DECLARATIONS

Conflict of interest

The authors declare no competing interests

Consent to publish

N/A

Ethical approval

N/A

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How to cite this article

Al-Dulymi HK, Alshaheen MR. Inorganic nanometal complexes as enzyme carriers for enhanced stability and biocatalysts. *Journal of University of Anbar for Pure Science*. 2026; 20(2):252-263. doi:[10.37652/juaps.2026.165452.1713](https://doi.org/10.37652/juaps.2026.165452.1713)