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Immobilization of *Penaeus merguiensis* alkaline phosphatase on gold nanorods for heavy metal detection

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ABSTRACT

Biotechnology of enzyme has gained popularity due to the growing need for novel environmental technologies and the development of innovative mass-production. The work describes the original application of biosensors based on *Penaeus merguiensis* alkaline phosphatase (PM ALP) immobilized on gold nanorods (GNRs) to heavy metal determination. *Penaeus merguiensis* alkaline phosphatase (PM ALP) was immobilized on gold nanorods (GNRs) by ionic exchange and hydrophobic interactions. The optimum pH and temperature for maximum enzyme activity for the immobilized PM ALP are identified to be 11.0 and 60 °C, respectively, for the hydrolysis of para-Nitrophenylphosphate (*p*-NPP). The kinetic studies confirm the Michaelis–Menten behavior and suggests overall slightly decrease in the performance of the immobilized enzyme with reference to the free enzyme. K_m and V_{max} values were 0.32 μm and 54 μm. min^{-1} for free and 0.39 μm and 48 μm min^{-1} for immobilized enzymes, respectively. Similarly, the thermal stability, storage stability and stability at extreme pH of the enzyme is found to increase after the immobilization. The inhibitory effect heavy metal ions was studied on free and immobilized PM ALP. The bi-enzymatic biosensor were tested to study the influence of heavy metal ions and pesticides on the corresponding enzyme. The obtained high stability and lower decrease in catalytic efficiency suggested the great potential and feasibility of immobilized PM ALP nanobiocatalyst in efficient and apply the biosensor in total toxic metal content determination.

1. Introduction

Heavy metal pollution in coastal zone is a serious issue leading to considerable environmental and ecological degradation. Heavy metals, when found in high concentration in aquatic habitat, accumulate in different organisms, damaging their tissues and suppressing growth. However, analysis of total metal content in water and sediment does not predict the toxicity of contaminants to biota. Hence, aquatic organisms are often used as both 'biomonitors' and 'bioindicators' of environmental pollution (Chakraborty et al., 2014). Heavy metal biomonitors need to conform to certain required characteristics, not least being metal accumulators. Use of a suite of biomonitors allows recognition of the presence and relative magnitude of different metal sources. Enzymes are recognized as useful bioindicators for metal pollution in seawater due to their sedentary lifestyle, considerable biomass, and easy identification. Enzymatic biosensors can be defined as an analytical device having an enzyme as a bioreceptor integrated or intimately associated with the physical transducer to produce a discrete or continuous digital electronic/optical signal that is proportional to the concentration of analyte present in the sample (Karunakaran et al., 2015). Enzyme biosensors employ the affinity and selectivity of catalytically active proteins, towards their target molecules. Typically, enzyme, usually immobilized on/within the surface of transducer-acts as a catalyst when interacting with the analyte, represented by its substrate, inhibitor, co-substrate or co-factor, while the enzyme itself remains unchanged (Nnewma and Setford, 2006; Gumpu et al., 2015). Depending on the assay type, two fundamental classes of enzyme sensors can be distinguished. First, the enzyme detects the presence of a substrate, or co-substrate/co-factor. This is then, by way of a transducer, used to monitor the increase of enzymatic activity. A typical example is a glucose biosensor (Karunakaran et al., 2015; Nnewma and Setford, 2006; Chen et al., 2013; Scognamiglio, 2013). The second group is based on the detection of inhibitors in the presence of a substrate (Ilangovan et al., 2006; Tekaya et al., 2013). With this system the decrease of signal (caused by enzyme inhibition) is monitored. The most common example of this approach is the detection of organophosphate compounds used as pesticides or warfare nerve agents (Syshchyk et al., 2015). Immobilization of enzymes is an important feature in designing the biorecognition part of enzyme based biosensors (Schmid, 2015). Enzyme immobilization appears as a key factor to develop efficient biosensors with appropriate performances such as good operational and storage stability, high sensitivity, high

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selectivity, short response time, high reproducibility, economic convenience, higher stability, and the possibility to be easily removed from the reaction mixture leading to pure product isolation (Homaei et al., 2013; Sassolas et al., 2012). The use of nanomaterials for the design of biosensing devices constitutes an exciting and recent approach to improve the performance of detection platforms. The extremely promising prospects of nanomaterials are due to their unique properties (Pogorilyi et al., 2016; Vopálenská et al., 2015). Several examples of nanostructures of different shape, metal and, possibly, assembled in ordered or random arrangements are reported in the literature, resulting in electromagnetic SERS enhancement factors up to 10^8. For example, rod-shaped metal nanoparticles possess two plasmon resonances, a transverse mode perpendicular to the long axis of the rod and a longitudinal mode parallel to the long rod axis. The latter depends linearly on the aspect ratio, i.e., the length divided by the width of the nanorods (NRs), and it is widely tunable in the visible and in the near infrared region of the spectrum (Ros et al., 2014; Mirza et al., 2016; Lin et al., 2016). In addition, both plasmon resonances depend on the aggregation state of the nanoparticles and on the refractive index of the surrounding medium. The possibility to tune the resonance wavelength can be exploited to amplify both laser and scattered field in SERS measurements at the desired wavelength (Ros et al., 2014). gold is a coinage metal that is known to exhibit surface enhancement of the Raman signal if the gold surface is covered by nanoparticles or is nanopatterned (Mirza et al., 2016).

Gold nanorods (GNRs) are rod-like shaped plasmonic nanoparticles with special optical properties because of their anisotropy. GNRs have three main advantages: (1) the SPR extinction in the near-infrared (NIR) region of GNRs provides opportunities in NIR photoabsorption and scattering and medical photothermal therapy; (2) the stronger longitudinal SPR band makes GNRs more sensitive to changes in size, shape, nano-environment, or interparticle distance; (3) the tunable SPR band can improve surface-enhanced Raman scattring sensing and imaging (Gui and Cui, 2012).

Gold nanorods (GNRs) with distinctive shape-dependent optical properties have drawn worldwide attention in the biomedical field. These nanorods possess two different plasmon bands. One is the transverse plasmon band in the visible region at around 520 nm, and the other is the longitudinal plasmon band in the near-infrared region (Stone et al., 2011; Huang and El-Sayed, 2010).

GNRs show strong optical absorption and their surface chemistry allows straightforward attachment of organic molecules tailored for specific purposes. Due to their unique nature, these rod-shaped nanostructures have rapidly found broad place in biological and biomedical applications including biosensing, drug delivery, photothermal therapy, and imaging. The sensitivity of the surface plasmon resonance (SPR) bands to the local environment is quite important in terms of biological sensing (Homaei et al., 2014). Many compounds used in different fields of industry and/or agriculture act as inhibitors of enzymes, which, as consequence, are unable to bind the substrate. Even if it is not so sensitive, the method for detecting heavy metal traces using biosensors has a dynamic trend and is largely applied for improving the “life quality”, because of biosensor's sensitivity, selectivity, and simplicity. In the last years, they also become more and more a synergetic combination between biotechnology and microelectronics (Turdean et al., 2011). Numerous enzyme have been utilized for heavy metal detection. Alkaline phosphatase is an enzyme of high research interest, having also a variety of industrial applications. In my previous research, the ALP of *Penaeus merguiensis* has been studied for the first time and a novel ALP was purified and characterized. It has been named as PM ALP. These data clearly indicate that the sensitivity of the enzyme increases against the inhibitory effects ions of the first transition series that are elements in marine pollution. Such inhibition may be due to masking of binding sites on the enzyme by these metal ions. The experimental results showed that all these metal ions also inhibited the activity of ALP from *Penaeus merguiensis*. Therefore, marine pollution might disrupt the production of *Penaeus merguiensis* (Homaei, 2015a). Biosensors based on the principle of enzyme inhibition have been applied for a wide range of significant analytes such as organo-phosphorous pesticides (OP), organo-chlorine pesticides, derivatives of insecticides and heavy metal ions (Van Dyk and Pletschke, 2011; Amine et al., 2016; Mehta et al., n.d.). The activity of PM ALP decreases in presence of metal ions and causes lowering of product formation. The extent of inhibition is directly proportional to the concentration of metal ions in solution (Songa and Okonkwo, 2016). A wide range of toxic analytes including heavy metals, pesticides, insecticides, and glycoalkaloids based of enzyme inhibition can be detected by electrochemical biosensors (Amine et al., 2016; Mehta et al., 2016). Immobilized enzyme offer tremendous scope for analytical purposes because they can be readily separated from reaction medium. Employment of alkaline phosphatase for assessment of heavy metal toxicity appears to be simple and sensitive tool for the quantitation; a method basically based on the strong inhibitory effect of these metal ions on alkaline phosphatase activity. A few works used alkaline phosphatase for biosensor analysis of heavy metals. In the current article, we report immobilization of PM ALP on GNRs for the first time. Alkaline phosphatase was immobilized on gold nanorods and conditions for the immobilization and characterizations of the immobilization enzyme were specified. The efficacy of immobilization was determined by measuring alkaline phosphatase activity and stability and comparing the data with those obtained utilizing its soluble form. The main objective consisted in studying the inhibition potency of several heavy metal ions and assessing the adequacy of the obtained enzyme system for the preparation of PM ALP biosensor for the determination of low concentrations of heavy metal ions. It is expected that the results presented in this paper would provide a sound basis to further investigations.

2. Experimental

2.1. Chemicals

Alkaline phosphatase was purified from *Penaeus merguiensis* brain (Homaei, 2015a). All chemicals were reagent grade and purchased from Merck (GmbH, Darmstadt, Germany).

2.2. Preparation, purification and characterization of gold nanorods

Short gold nanorods were synthesized via sequential seed mediated growth method, as described previously (Homaei et al., 2014). The sample previously purified by three rounds of centrifugation (12,000 rpm for 3 min), was redispersed in deionized water. Dilute sample was deposited on a carbon coated copper grid and left undisturbed for solvent evaporation. Gold nanorods were also characterized by UV–Vis absorption spectrophotometer (Perkin Elmer Lambda 25) in the 400–850 nm. The morphology of rod was confirmed by UV–Vis spectroscopy. Zeta potential measurements of the particles were carried out on a Malvern Zeta Sizer (Nano ZS) at room temperature (Homaei et al., 2014).

2.3. Immobilization of Penaeus merguiensis alkaline phosphatase on gold nanorods

5 mg ml^{-1} enzyme solution in a mixed buffer containing 50 mM each acetate, phosphate and glycine, pH 8.0, was incubated with 30 nm purified GNRs at room temperature for 2 h. The enzyme bound to gold nanorods surface was measured for determining the enzyme concentration in supernatant after immobilization. The loss in absorbance at 280 nm in the supernatant was used to quantify the amount of bound enzyme on gold nanorods. All samples were kept refrigerated until assay.

2.4. Determination of alkaline phosphatase activity and protein concentration

Phosphatase activity was determined in Alkaline phosphatase buffer, containing 50 mM Tris-HCl, 100 mM NaCl and 2 mM $MgCl_2$ at pH 11 and room temperature, using 2 mM para-Nitrophenylphosphate (*p*-NPP) as substrate. *p*-NPP is a chromogenic substrate for acid and alkaline phosphatase in ELISA and conventional spectrophotometric assays. These enzymes catalyse the hydrolysis of *p*-NPP to yellow para-nitrophenol. This product absorbs strongly at 405 nm and can be measured with a visible light spectrophotometer. To 100 µl of enzyme solution diluted in 500 µl of ALP buffer, 400 µl of 2 mM *p*-NPP was added and the reaction mixture was incubated at room temperature for 5 min. The hydrolysis of *p*-NPP to *p*-NP (a chromogenic product with absorbance at 405 nm) in ALP buffer was measured for 5 min. The reaction was stopped by adding 500 µl of 1 M NaOH and the amount of *p*-NP liberated was estimated by measuring its absorbance at 405 nm in a Perkin Elmer's LAMBDA 850 UV/Vis Spectrophotometer (Homaei, 2015a). One unit of phosphatase is defined as the amount of enzyme that hydrolyzes *p*-NPP to produce equivalent absorbance to 1µmol of *p*-NP/min. In the case of the immobilized ALP, the reaction mixture was continuously stirred during the reaction. Protein concentration was estimated by the Bradford method using bovine serum albumin as standard.

2.5. Activity and stability studies on free and immobilized alkaline phosphatase

The activity versus pH profiles of free and immobilized enzyme were graphed measuring the alkaline phosphatase activity at room temperature in a mixed buffer containing 50 mM acetate, phosphate and glycine in the range 4–12 pH values. The stability to pH was checked after incubation of the enzyme in 50 mM of mixed buffer pH 3 and 12 for different intervals of time at room temperature, then pH value was adjusted to 11.0 and the residual activity determined according to the assay conditions. Control measurements were carried out measuring the activity of the same enzyme solution kept in the buffer at pH 11.0. The activity of free and immobilized enzyme versus temperature profiles were graphed on the basis of the activity values measured at different temperatures in the range 20–90 °C, in alkaline phosphatase buffer of pH 11.0, as described above. To determine the thermal stability, free and immobilized alkaline phosphatase were incubated at 60 and 70 °C in 50 mM in Na_2CO_3–$NaHCO_3$ buffer pH 11.0, for different intervals of time, then cooled on ice and the residual activity determined under the assay conditions. Control measurements were carried out measuring the activity of the same enzyme solution kept on ice.

2.6. Determination of storage stability

The storage stability of free and immobilized alkaline phosphatase periodically was investigated by measuring their residual activity after incubation on 4 °C and room temperature under assay conditions, every 10 days for a period of 90 days.

2.7. Determination of kinetic parameters

Catalytic activity of free and immobilized alkaline phosphatase were investigated at different *p*-NPP concentrations under assay conditions. K_m, V_{max}, K_{cat} and K_{cat}/K_m values were determined using Michaelis-Menten plots.

The results are mean value of three independent experiments and they were repeated for reproducibility.

2.8. Effect of heavy metal ions on enzyme activity

Effect of metal ions on the free and immobilized enzyme was studied by adding a special metal ion into the assay system containing 2 mM *p*-NPP and 50 mM Tris–HCl buffer at pH 11 and room temperature.

3. Results and discussion

3.1. Alkaline phosphatase immobilization and characterization of Nanorods-enzyme interaction

Immobilization of ALP on GNRs was carried out simply by mixing a relative concentrate solution of the enzyme with GNRs suspension at room temperatures with constant shaking at 200 rpm for 4 h. Longer incubation time did not affect the surface coverage. Mixture was then centrifuged at 12,000g at 25 °C for 5 min. The supernantant was removed and GNRs were washed with Tris-HCl buffer. The PM ALP adsorbed on GNRs were dispersed in 1 ml of Alkaline phosphatase buffer, containing 50 mM Tris-HCl, 100 mM NaCl and 2 mM MgCl2 at pH 11. The ALP activity was determined in the GNRs and in supernatant and washings. The difference between the total enzyme activity loaded and the total activity in the supernatant and washings represent the theoretical amount of enzyme activity bound (A). The enzyme activity obtained in GNRs represents the actual expressed enzyme activity (B). The efficiency factor of the immobilization of the biocatalyst was calculated by dividing the actual enzyme activity expressed (B) by the enzyme activity bound (A) i.e. efficiency factor, η=B/A (Bickerstaff and Taylor, 1992).

Zeta potential value of the nanorods, measured at room temperature by electrophoretic light scattering technique, was found +41.0 mV, a value that indicates a good stability of the colloidal dispersion (Homaei and Etemadipour, 2015a). ALP adsorption produces a slight change in the longitudinal surface plasmon resonance (LSPR) intensity of GNRs (Fig. 1). The main initial driving force for the adsorption of enzyme to GNRs is of electrostatic nature between the positively charged surface of the nanorods and the negatively charged enzyme (Ghiaci et al., 2009; McLaren and Packer, 2006). Furthermore, cetyl residues, characterized by a 15 methylene length chains, create a highly hydrophobic environment which can favor interactions with hydrophobic zones of the enzyme, due to the presence of solvent accessible non-polar amino acid residues in the primary structure (Radi et al., 2006).

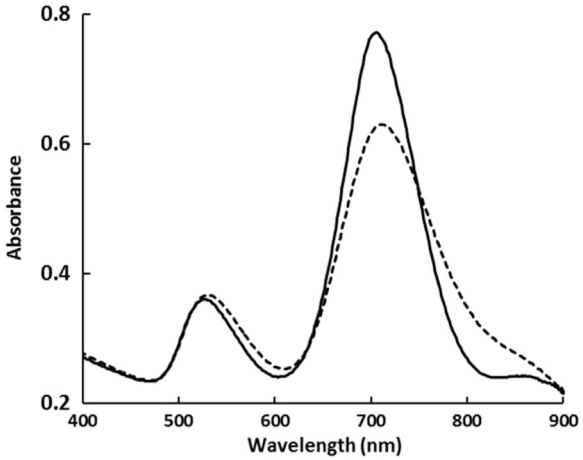

Fig. 1. Absorption spectra of 30 nm purified gold nanorods (solid line); linked to 5 mg ml^{-1} alkaline phosphatase (dotted line)..

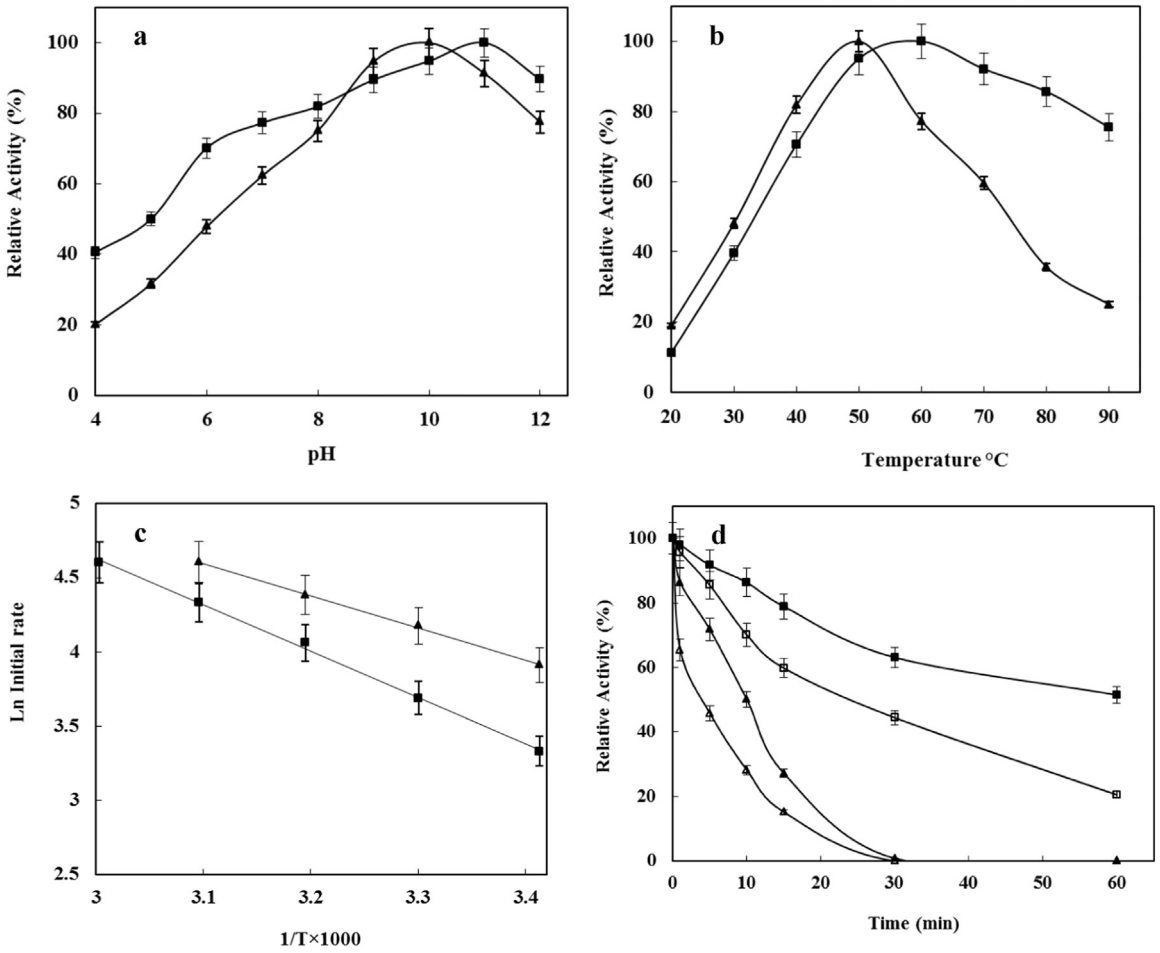

Fig. 2. a) Effect of pH on the activities of free (▲) and immobilized (■) enzyme. The activity at optimal pH was taken as 100%. **b)** Effect of temperature on the activities of free (▲) and immobilized (■) enzyme. The activity at optimal temperature was taken as 100%. **c)**The respective Arrhenius plots of free (▲) and immobilized (■) enzyme.are reported. **d)** Irreversible thermoinactivation of free (▲) and immobilized (■) ALP at 60 °C **(**solid**)** and 70 °C **(**hollow**)**. The activity of the same enzyme solution, kept on ice, was considered as the control (100%).

3.2. Characterization of free and immobilized enzyme

3.2.1. The pH effect on activity

Effect of pH on the activities of free and immobilized alkaline phosphatase samples were investigated at room temperature in the pH range 4.0–12.0 and results were given in Fig. 2a. ALPs immobilized on GNRs showed a broader profile than that of the free ALP, and the ALP activity was preserved in a wider pH range. Free ALP exhibits maximum activity at a pH of 10. As can be seen, the optimum pH of the resultant GNRs–ALP bioconjugates shifted from 10.0 to 11.0, which was mainly due to the charge changes of the GNRs surface during the immobilization process (Homaei et al., 2014; Homaei and Etemadipour, 2015b). Depending on the charge properties of the matrix, the optimum pH may undergo significant shifts. Generally optimum pH of many immobilized enzymes moved to more alkaline values if the net potential of the carrier is negative and to more acidic values if it is positive, due to the change in the degree of ionization of the functional groups of the active centers (Ghiaci et al., 2009; McLaren and Packer, 2006; Palmer, 1995). The profile of immobilized enzyme shows higher activity both in acidic and basic pH ranges compared to its free form, values of relative activity higher than 70% in the pH range 6.0–12.0 were found. Furthermore, pH profiles of the immobilized ALP displayed significantly improved stability in basic range of the pH compared to that of the free form. These results show that the adsorption enzymes on GNRs preserves enzyme activity over a wider pH range, which is in agreement with other studies (Homaei et al., 2014; Homaei and Etemadipour, 2015b).

3.2.2. Effect of temperature on activity

The influence of temperature on the activity of free and immobilized enzyme was examined (Fig. 2b). A significant shift to higher temperature was observed upon immobilization. Maximum activity for free and immobilized PM ALPs was observed at 50 and 60 °C respectively. Moreover, the immobilized ALP is active in a broad temperature range. It exhibited more than 60% of maximum activity in the range of 40–80 °C. At 90 °C, the immobilized ALP displayed more than 75% of maximum activity, while it was 25% in the case of the free form. The Arrhenius plots were graphed utilizing the activity values in the temperature range of 20–60 °C and 20–70 °C for ALP and its immobilized forms, respectively (Fig. 2c). activation energy values of 8.3 and 5.2 kcal mol^{-1} K^{-1} for free and linked enzyme, respectively, were obtained. These results suggest a decrease in sensitivity to temperature and an increase in conformational rigidity of the immobilized enzyme compared with free form. Apparently, the E_a of ALP decreased after being immobilized, which indicated that the catalytic reaction ratio increased and it was less sensitive to temperature under the same reaction condition. Hence, immobilization improved the quality of ALP by lowering down the energy required to make the activated complex (ES).

3.2.3. Effect of immobilization on thermostability

Irreversible thermoinactivation of free and immobilized ALP was determined at 70 and 80 °C. As shown in Fig. 2d, the immobilized ALP exhibited a marked increase in thermostability as compared with its free counterpart. The half lives of free enzyme were 10 and 7 min at 70

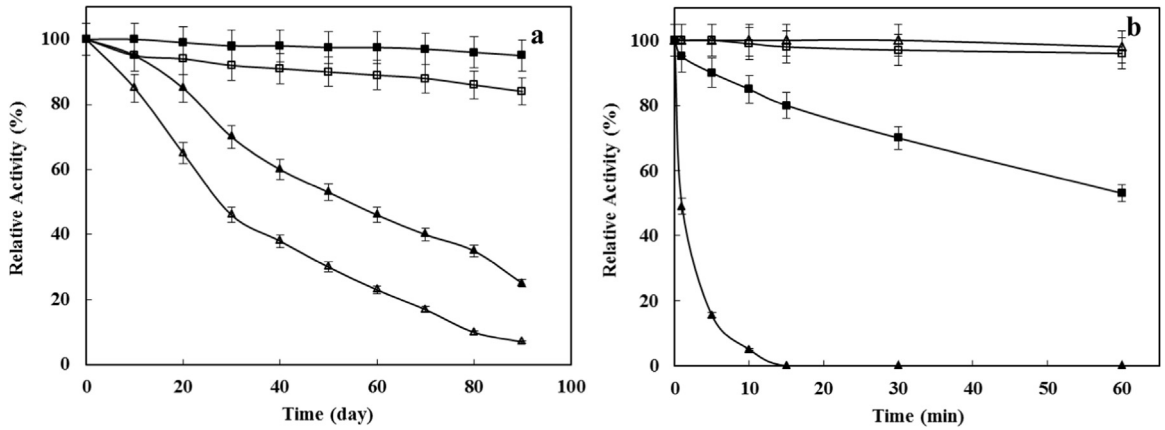

Fig. 3. a) Storage stability of free (triangles) and immobilized (squares) ALP at 4 °C (solid) and at room temperature (hollow). **b**) Irreversible inactivation of free (▲) and immobilized (■) ALP after incubation for different times at pH 3.0 **(**solid**)** and 12 **(**hollow**)**. The activity of untreated enzyme was taken as 100%.

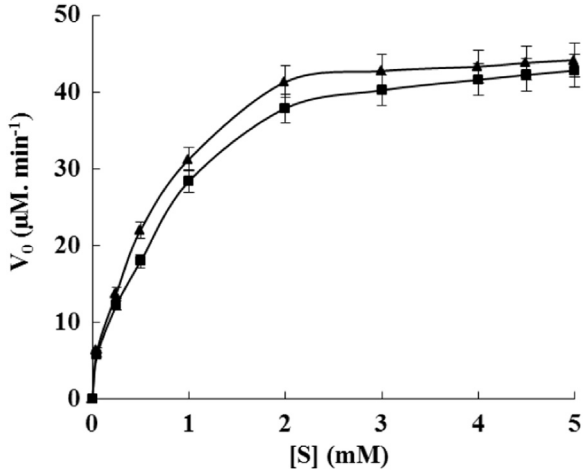

Fig. 4. Michaelis-Menten plots for the *p*-NPP hydrolysis with free (▲) and immobilized (■) Alkaline phosphatase.

and 80 °C respectively, while the half life of immobilized enzyme was more than 60 and 40 min at 80 °C. When incubated at 60 °C for 90 min, the free ALP was totally denatured, while the immobilized enzyme still retained more than 50% of its initial activity. Immobilization of ALP on GNRs caused an increase in conformational rigidity of protein structure and limited its freedom to undergo drastic conformational changes. This resulted in an increased stability towards thermal denaturation (Homaei et al., 2014; Homaei and Etemadipour, 2015b; Zhang et al., 2014; Tiwari et al., 2011). In industrial processes, it can be an advantage to use enzymes that are operationally stable at high temperature as industrial processes require a rise in.

temperature for more efficient substrate release/conversion (Turner et al., 2006; Chen et al., 2014).

In addition to improving thermal stability, the storage stability of alkaline phosphatase exhibited a significant increase upon immobilization. As indicated in Fig. 3a, free alkaline phosphatase was remained 25% and 7% of its initial activity after 90 days of incubation at 4 °C and room temperature respectively. While under these condition, 95% and 85% of its original activity remained after 3 months for immobilized alkaline phosphatase at 4 °C and room temperature respectively. According to these results, the activity of enzyme was usually declined after a long time. Therefore, immobilization significantly prevented this event and improved the storage stability of enzyme.

3.2.4. *Effect of immobilization on extreme pHs stability*

The stability of free and immobilized ALP was measured at two extreme pH's 3.0 and 12. As shown in Fig. 3b, the immobilized enzyme was more stable as compared with free enzyme not only at pH 12 but also at pH 3. After 60 min of incubation at these pH values, the immobilized ALP retained about 53% and 98% of its residual activity at pH 3 and 12, respectively. Under the same conditions at pH 3, a nearly complete inactivation of free ALP was observed. This result indicated that the immobilization appreciably enhanced the stability of ALP both in the acidic and basic pHs whereas the free ALP was sensitive to extreme pHs. Mechanisms of the decreased free ALPs activity might be attributed to the changed protein folding and binding modes (electrostatic or via hydrogen bonds) (Wu et al., 2011). On the other hand, stabilization of ALP on GNRs may occur through multipoint no-covalent electrostatic and hydrophobic interactions. These types of interactions which increase conformational rigidity and stabilize globular steric structure against particular environmental conditions is able to denaturate proteins from the native form (Homaei and Saberi, 2015; Cao et al., 2014; Komathi et al., 2013; Copeland, 2000).

3.2.5. *Effect of immobilization on kinetic parameters*

Catalytic kinetics of the free and immobilized enzymes were further investigated. possibly caused by the immobilization were further investigated. Michaelis-Menten plot for *p*-NPP reduction (0–2 mM) with free and immobilized PM ALPs is shown in Fig. 4. The apparent maximum steady-state rate, V_{max}, the apparent Michaelis constant, K_m, and apparent catalytic constant k_{cat} values of the immobilized and free ALPs calculated from the equations of these plots were summarized in Table 1. As we know, The K_m is the substrate concentration that provides a reaction velocity that is half of the maximal velocity obtained under saturating substrate conditions that depends upon both partitioning and diffusional effects. The value of k_{cat} is sometimes referred to as the turnover number for the enzyme, since it defines the number of catalytic turn over events that occur per unit time. Turnover numbers, however, are typically reported in units of molecules of product produced per unit time per molecules of enzyme present. V_{max} is the maximum velocity or rate at which the enzyme catalyzed a reaction (Copeland, 2000). As indicated in Table 1, The K_m value of the immobilized enzyme was higher than that of the free enzyme, and the V_{max} value of the immobilized enzyme was lower than that of the free enzyme. The above results imply that the immobilized enzyme has an

Table 1
Kinetic parameter s for free and immobilized FM Alkaline phosphatae.

Enzyme form	V_{max} (µM min^{-1})	k_{cat} (s^{-1})	K_m (µM)	k_{cat}/K_m (µM^{-1} s^{-1})
Free[a]	54 ± 0.42	97 ± 0.37	0.32 ± 0.72	303 ± 0.92
Immobilized	48 ± 0.83	93 ± 0.58	0.39 ± 0.20	238 ± 0.43

[a] Data presented for the free enzyme have already been measured in ref (Homaei et al., 2014).

apparent lower affinity between enzyme and substrate, suggesting that it was more difficult for the substrate to diffuse and access the enzyme and that a higher concentration of substrate is needed when utilizing the immobilized enzyme (Garcia-Galan et al., 2011; Chang and Juang, 2005). The lower affinity due to the negative effect of immobilization in terms of increased steric hindrance of the active site or substrate diffusion resistance. Moreover, the lower affinity of the immobilized enzyme caused by the loss of enzyme flexibility necessary for substrate binding (Garcia-Galan et al., 2011; Chang and Juang, 2005; Long et al., 2015).

A slightly decrease in k_{cat} of immobilized compared with its free counterpart just due to the active sites of the enzyme are oriented toward the reaction mixture and slight distortions of the enzyme structure associated to the immobilization (Homaei, 2015b). In addition, the catalytic efficiency defined by the k_{cat}/k_m ratio showed a lower value for immobilized ALP compared to free enzyme. Nevertheless, this slight decrease did not significantly influence their catalytic efficiency. This could be due to the difference in size and surface chemistry of the nanostructures, since the modified rods have been synthesized with least required concentration of the surfactant in the presence of more silver ions (Homaei et al., 2014; Homaei and Saberi, 2015).

3.2.6. Immobilized enzyme on gold nanorods as a potential enzymatic biosensor for determination of heavy metal ions

Relative activities of PM ALP and its immobilized form in the presence of different concentrations of various bivalent metal ions are depicted in Table 2. The free enzyme appears strongly inhibited by Hg^{2+}, Cu^{2+} and Pb^{2+}, In particular, Hg^{2+} concentration higher than 1 mM is sufficient to totally inhibit alkaline phosphatase activity, while a value lower than 2 mM is requested in the case of Cu^{2+} and Pb^{2+}. From the experimental data, the following scale of alkaline phosphatase inhibition from bivalent ions can be drawn up: $Hg^{2+} > Cu^{2+} > Pb^{2+}$(Homaei, 2015a). Inhibition data by heavy metal ions clearly indicate that the sensitivity of enzyme is reduced owing to immobilization process (Table 2). Analogously to the stability to pH, lower sensitivity of immobilized enzyme toward heavy metal ions can be due to the GNRs surface characterized by diffuse positive charges that can drive back divalent ions creating a microenvironment around the enzyme characterized by a lower ion concentration (Homaei et al., 2014). Such protection may be due to masking of binding sites on the enzyme as well as the diffusion limitations. This inhibition reduced by partial blocking or just by inducing a certain distortion in the inhibition site by immobilization (Homaei and Etemadipour, 2015b). The immobilization of PM ALP on the GNRs likely altered the molecular conformation of the enzyme, making it difficult for metal ions to access the active sites and to attack PM ALP, or simply blocked the active sites. Thus, a higher activity of a given enzyme after immobilization, in some instances, derived from a decrease in enzyme inhibition and not from the production of a more active conformation of the enzyme (Homaei and Etemadipour, 2015b).

Immobilized Alkaline phosphatase present in the brain cells of *Penaeus merguiensis* can be used as a tool for the detection of heavy metal ions. The potential uses of PM ALP include in the enzyme inhibition-based biosensors for food safety and environmental monitoring. The bi-enzymatic biosensors were tested to study the influence of heavy metal ions and pesticides on the corresponding enzyme. Additionally, The PM ALP was immobilized on GNRs surface and further incorporated with a conductivity transducer to determine the inhibitory effect of phosphate on the immobilized enzyme. The developed biosensor can be used for its potential application in determining the phosphate level of serum and tap water. Moreover as enzymatic activity inhibited by different families of pollutants can be detected, this biosensor can be helpful for further lab analysis with conventional analytical techniques. Using whole cells is also particularly interesting since ecotoxicological parameters can be integrated, especially the true toxicity of a compound for an organism (Chouteau et al., 2005).

Table 2
Effect of various chloride metal ions and their concentrations on free and immobilized *Penaeus merguiensis* alkaline phosphatase. The enzyme activity at zero concentration of each ion was taken as 100% and the other concentrations were measured with respect to zero concentration. Measurements were carried out in substrate saturation conditions.

[Me^{2+}] (mM)	Relative activity (%)	[Me^{2+}] (mM)	Relative activity (%)
Free		Immobilize	
Hg^{2+}		Hg^{2+}	
1	6 ± 0.7	1	74 ± 0.3
2	0	2	56 ± 0.5
3	0	3	42 ± 0.6
4	0	4	31 ± 0.1
5	0	5	19 ± 0.8
Pb^{2+}		Pb^{2+}	
1	38 ± 1.3	1	96 ± 0.7
2	0	2	91 ± 0.2
3	0	3	83 ± 0.6
4	0	4	74 ± 0.5
5	0	5	69 ± 0.7
Cu^{2+}		Cu^{2+}	
1	20 ± 0.5	1	82 ± 0.4
2	0	2	67 ± 0.1
3	0	3	52 ± 0.8
4	0	4	44 ± 0.6
5	0	5	36 ± 0.4

4. Conclusions

Despite the ever-increasing number in enzyme inhibition based biosensors in the last decades, some issues still raise a barricade in their broader area of application. Sensitivity of the enzyme inhibition biosensors for the compound other than the analyte to be assayed is the main problem (Bachan Upadhyay and Verma, 2013). Taking into account the growing use of enzyme inhibition based biosensors, we can make that the catalytic action of the enzyme is inhibited by the presence of a given inhibitor species in the medium. The enzyme inhibition methods reported so far are seldom applied to real samples and, in some cases; a separation step precedes the enzyme inhibited reaction (Kuswandi and Mascini, 2005).

In this research, we explored the excellent adsorption of a ALP from *Penaeus merguiensis* on GNRs by ionic and hydrophobic interactions. The combined effect of broader temperature and pH profile, increased thermal stability as well as stability at critical pHs and improved storage characteristics highlight the value of GNRs as a support for ALPs immobilization. Compared with the kinetic parameters of free enzymes, the immobilized enzymes exhibited similar biocatalytic behavior evidenced from their similar K_m, k_{cat} and V_{max} which indicated that the GNRs support did not significant hinder the mass transfer. This implies that immobilization had a positive effect on the thermal stability of ALP and was able to protect the active conformation of the enzyme from damage. The immobilized enzyme better tolerated the effects of heavy metal ions when compared with free enzyme, making it more useful under complex conditions.

The advantages of such a system are the simplicity of the procedures for inhibition measurments and data processing, and its applicability for monitoring toxicity of natural water samples. This work has shown that a simple and rapid biosensor can be developed in future for monitoring of heavy metal ions, based on the inhibition of alkaline phosphatase activity. However, further work is necessary before considering these biosensors as competitive tools for on line and in situ monitoring that can be used as early warning systems for qualitative analysis. With this stabilizing effect, immobilized ALP exhibits an advantage in continuous applications for the determination of heavy metals in environmental compartments.

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