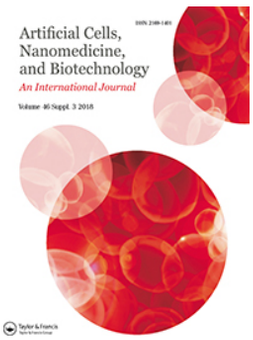

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Immobilization of horseradish peroxidase on PMMA nanofibers incorporated with nanodiamond

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ABSTRACT

In the present study, nanodiamond (ND) was blended with polymethyl methacrylate (PMMA) and then electrospun into nanofibers (nfPMMA-ND) for the immobilization of horseradish peroxidase (HRP). The maximum immobilization efficiency of HRP (96%) was detected at 10% ND and pH 7.0. ATR-FTIR, SEM and TEM were used to characterize the immobilized enzyme. The immobilized enzyme retained 60% of its initial activity after ten reuses. The pH was shifted from 7.0 for soluble HRP to 7.5 for the immobilized enzyme. The soluble HRP had an optimum temperature of 30 °C, whereas this temperature was shifted to 40 °C for the immobilized enzyme. The substrate analogs were oxidized by immobilized HRP with higher efficiencies than those of soluble HRP. The kinetic results showed that the soluble HRP had more affinity toward guiacol and H_2O_2 than immobilized HRP. The effect of metal ions on soluble and immobilized HRP was studied. The immobilized HRP was markedly more stable when it exposed to urea, isopropanol, butanol and heptane compared with the soluble enzyme. The immobilized HRP exhibited high resistance to proteolysis by trypsin than that of soluble enzyme. In conclusion, the nfPMMA-ND-HRP could be employed in several applications such as biosensor, biomedical and bioremediation.

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KEYWORDS
Nanodiamond; horseradish; peroxidase; immobilization

Introduction

Nanostructures have emerged as a new attractive technique for enzymatic immobilization processes, since they possess unique characteristics to equilibrate principal factors which enable the determination of biocatalysts efficiency, including specific surface area, mass transfer resistance, and effective desired enzyme loading. Many materials at the nanoscale have been utilized in the processes of immobilization, such as silicon, gold, diamond, carbon nanotubes and graphene. The nanostructured materials provide ideal characteristics for maintaining enzyme stability and have some advantages over conventional immobilization supports. The functionalization of these supporting materials is excessively studied [1]. Enzymes are very specific biocatalysts, which may have their utilization enhanced and improved by immobilization. In the industrial and biomedical applications, it is necessary to immobilize the enzymes to prolong its catalytic activities [2]. This process will provide the enzyme with more stability and resistance to severe conditions, including ranges of pH, temperature and preservation of the enzyme activities through several cycles [3]. Among the wide range of nanomaterials, detonation ND (i.e. ultradispersed ND with particle size in the range 3–10 nm) came into focuses as a potential useful material for biomedical applications due to its unique physical and chemical properties, such as small size, chemically active surface area, and stability toward corrosive conditions [4]. Since the surface of the graphitic shell of the ND can be functionalized with a variety of functional groups including carboxyl, lactone, ketone, hydroxyl and some alkyl groups. Thus, ND may be easily activated for conjugation with biological molecules. Also, the small particle size of the ND has a positive effect on the immobilized enzyme activity [5]. It has been reported that the surface carboxylic groups present in ND could easily bio-conjugated with enzyme using mild coupling agents (1-ethyl-3–(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) [5]. Nguyen et al [6] demonstrated that carboxylated/oxidized diamond crystallites can be utilized as a solid supporting material for non-covalent protein immobilization.

Horseradish peroxidase (HRP), a glycoprotein with molecular mass 40 kDa, is one of the most extensively studied

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enzymes because of its wide spectrum of applications. HRP has been utilized for the removal of phenols from wastewater [7], organic compound syntheses [8] and applications for analytical purposes [9]. Investigations of HRP have been developed because of its high activity, simple detection of products, relative stability, ease of immobilization and the stability of the immobilized preparations [9]. Polymethyl methacrylate (PMMA) and cotton were used for immobilization of HRP as H_2O_2 sensor [10]. In this work, we immobilized HRP on PMMA nanofibers scaffold incorporated with ND. It is known that nanofibers scaffold has the advantage of a high surface area, therefore, it was envisioned that having both PMMA (dissolved) and ND dispersed in dimethylformamide (DMF) would allow making nanofibers scaffold that contains ND. Since ND has reactive amino, carboxylic and hydroxyl groups, thus it is expected that these functional groups would assist in binding with an enzyme. To the best of our knowledge the immobilization of HRP via non-covalent bond on PMMA treated with ND has not yet been reported. Therefore, in this study, an effort has been made to immobilize HRP on PMMA treated with ND. The biochemical properties of immobilized HRP have been evaluated and compared with those of the soluble HRP.

Materials and methods

Horseradish peroxidase

Horseradish peroxidase (HRP) was previously purified from horseradish cv. Balady. The detailed procedure was reported in our previous paper [11].

Chemicals

PMMA (Avg Mw 996000) from Aldrich Chemical Co., Inc., and detonation ND 6 nm APS (~98% purity) from Nanostructured & Amorphous Materials Inc, USA, were used as received. N,N-dimethylformamide of Puriss. P.a., ACS reagent, reag. Ph. Eur., >99.8% (GC) and Acetone of Puriss.P.a., ACS reagent, reag.ISO, reag. Ph. Eur., >99.5% (GC) from Aldrich Chemical Co., Inc., were used as such without further purification.

Preparation of nfPMMA-ND

ND was dispersed in DMF and acetone using ultrasonic bath CPX5800H-E, USA, 160 W (40 kHz)-6% for one hour. The resulting dispersions were homogeneous and stable, having a gray appearance. The required amount of PMMA was added to the ND dispersion and stirred using magnetic stirrer for ~24 h. The concentration of the polymer in the mixture was maintained in between 5 and 10 wt% so that the solution can be easily electrospun. Solutions with different loadings of ND were prepared by adding a constant weight of PMMA in the ND dispersion.

Electrospinning

The electrospinning process was carried out at room temperature. The solution was taken inside a syringe with the needle of 0.5 mm in diameter, the flow rate of 1.2 ml/h at the electric field strength of 21.5 KV/cm, distance 15 cm. Oriented fibers were obtained on different substrates (aluminum foil, a glass slide) placed over a stable flat surface.

Immobilization procedure

Different concentration of enzyme immobilization was carried out by end over end on the treated polymer (PMMA with 1, 5, 10, 15 and 20% ND) using a solution of HRP (831 units which represented 62,954 unit/mg protein) made in 50 mM sodium acetate buffer (pH 6) or Tris–HCl (pH 7.0 or 8.0) at room temperature during overnight. Aliquots of the supernatant were drawn up and the treated polymer was dried at room temperature to verify the advancement of the immobilization. The relative activity % of immobilized enzyme was calculated from the following formula

$$\text{Relative activity } \% \text{ of immobilized enzyme} = \text{Activity of immobilized enzyme}/\text{Initial activity of soluble enzyme} \times 100.$$

ATR-FTIR analysis

The attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectra for all samples were performed on a PerkinElmer spectrum 100 FT-IR spectrometer (PerkinElmer Life and Analytical Sciences 710 Bridgeport Avenue Shelton, CT 06484–4794 USA).

SEM and TEM analysis

FESEM images were taken out using a field emission scanning electron microscope (FESEM, Sirion-100, and FEI, USA). Samples on aluminum foil were spurted with Au for 0 0.5 min to make them conducive for FESEM observation. Transmission electron microscopy (TEM) characterization was carried out on an HT-7700 instrument (JEM-1200EX, Japan).

Peroxidase assay

Peroxidase activity was assayed according to Yuan and Jiang [12]. The reaction mixture contains in 1 ml: 8 mM H_2O_2, 40 mM guaiacol, 50 mM Tris-HCl buffer, pH 7.0and soluble HRP or immobilized HRP. The change of absorbance at 470 nm due to guaiacol oxidation was followed at 1 min intervals. One unit of peroxidase activity is defined as the amount of enzyme which increases the O.D. 1.0 per min under standard assay conditions.

Biochemical characterization of soluble and nfPMMA-ND peroxidases

Reusability of immobilized HRP

Reusability tests of the immobilized-HRP were carried out under standard conditions of enzyme assay described above. The product was measured at 470 nm after 1 min of reaction. Then, the immobilized -HRP was washed by 50 mM Tris-HCl buffer pH 7.0 and separated from the reaction medium. The immobilized-HRP recovered by this procedure was used repeatedly. The activity determined for the first time was considered as control (100%) for the calculation of remaining percentage activity after each use.

Effect of pH on soluble and immobilized-HRP

The activities of soluble and immobilized-HRP preparations were measured in buffers of various pH values (sodium acetate buffer pH (4.0–6.0) and Tris–HCl buffer pH (7.0–9.0).

Effect of temperature on soluble and immobilized-HRP

For measuring optimum temperature, the activities of soluble and immobilized-HRP Preparations were measured at various temperatures (30–80 °C) under standard assay conditions. The thermal stability was carried out by pre-incubating the reaction mixture at various temperatures (30–80 °C) for 15 min prior the substrate addition, followed by cooling in ice bath. The activity of enzyme at zero time was then taken as 100% activity.

Kinetic properties

The kinetic constant value (Km) was determined by measuring the initial reaction rates of soluble and immobilized-HRP using different concentrations of guaiacol and H_2O_2 as substrates.

Metal ion effect

The effects of various metal ions (Co^{2+}, Ca^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+}, Hg^{2+}, Cu^{2+}, Fe^{3+}, Cd^{2+}) on enzyme activity of soluble and immobilized-HRP were determined by pre-incubating the enzyme with 5 mM metal ions for 15 min and then assaying the enzyme activity. The activity in absence of metal ions is taken as 100%.

Effect of chemical compounds

The effect of urea, isopropanol, butanol and heptane on the enzyme activity of soluble and immobilized-HRP was determined by assaying the enzyme in the presence of these compounds. The activity in the absence of these compounds was taken as 100%.

Effect of trypsin

Soluble and immobilized-HRP preparations were incubated for 15 min with 5–25 mg trypsin/ml and then assaying the enzyme activity. The activity in the absence of trypsin was taken as 100%.

Table 1. The effect of nanodiamond concentration, which adsorbed on PMMA nanofibers and pH medium on the immobilization efficiency of HRP.

	Immobilization efficiency%		
Nanodiamond %	pH 6.0	pH 7.0	pH 8.0
0	8	16	12
1	23	63	42
5	25	45	33
10	42	96	62
15	17	41	28
20	9	39	16

Each value represents the mean of two experiments.

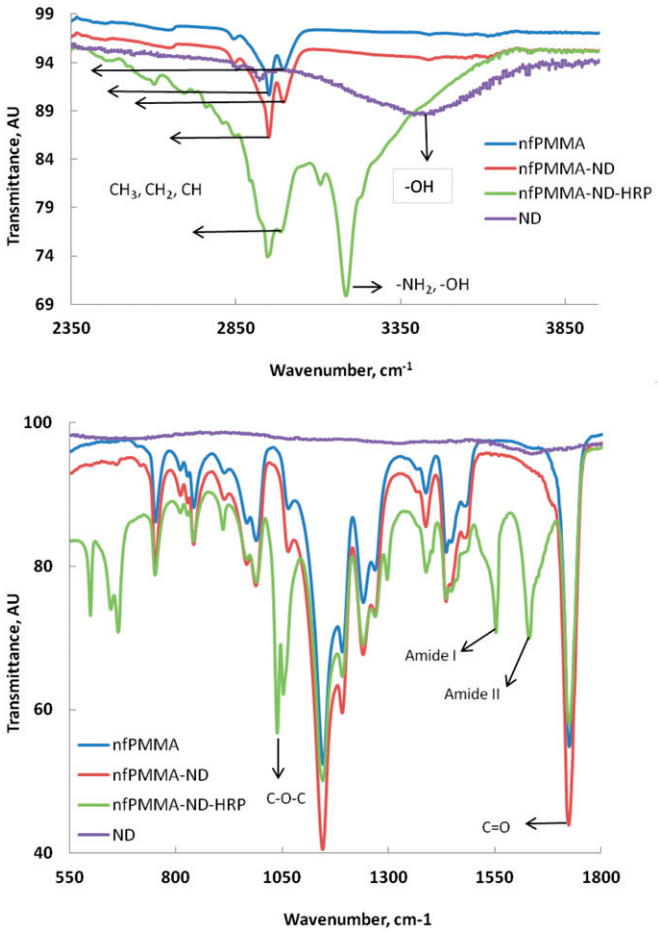

Figure 1. ATR-FT-IR spectra of ND, nfPMMA, nfPMMA-ND and nfPMMA-ND-HRP samples.

Results and discussion

The immobilization of HRP on nfPMMA polymer with different concentrations of ND at pH's 6.0 or 7.0 or 8.0 was studied. The maximum immobilization efficiency of HRP (96%) was detected at 10% ND and pH 7.0 (Table 1). The lower immobilization efficiency of HRP was detected at 20% ND and pH 6.0 or pH 8.0. The lowering in the immobilization efficiency of the enzyme at a high concentration of ND could be attributed to the presence of multipoint attachments of the enzyme to the treated nfPMMA polymer which lead to change in the structure of the enzyme. Several papers reported the high concentration of coupling reagent decreased the retained of the enzyme activity [13,14].

The source of ND is very much affecting its surface functionality as different sources would bring different chemical

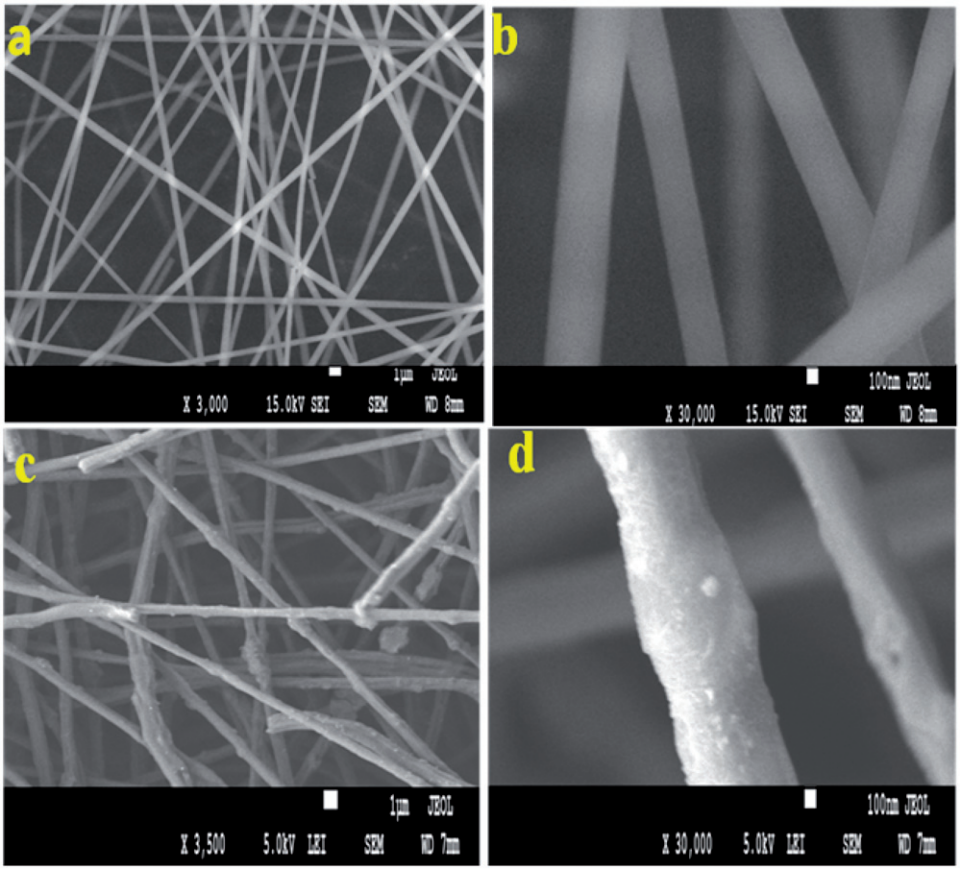

Figure 2. SEM Images of (a) nfPMMA low magnification (b) nfPMMA high magnification (c) nfPMMA-ND low magnification (d) nfPMMA-ND high magnification.

groups on the surface [15]. The ATR-FTIR spectra of the ND, nfPMMA, nfPMMA-ND and nfPMMA-ND-HRP samples are shown in Figure 1. The ATR-FTIR of the pristine ND used in the present work reveals peak at 3420 cm^{-1} owing to O–H on the surface and/or water adsorbed. Other characteristic peaks owing to C–H vibration appeared at 2850 cm^{-1} and 2920 cm^{-1}. Peaks in the range 1500–1650 cm^{-1} represented C=C vibrations [16,17]. On the other hand, nfPMMA sample showed the characteristic peaks of PMMA [18]. Peaks at 2994, 2950 together with 2840 cm^{-1} were due to the stretching vibration of C–H present in $–CH_3$ and $–CH_2$- groups, respectively. A sharp characteristic peak due to carbonyl ester is shown at 1724 cm^{-1}. Very intense peaks from 1145 to 1240 cm^{-1} are due to C–O–C stretching vibration. Peaks at 1435 and 986 cm^{-1} might be due to C–H bending vibration of the $–CH_3$ group. Other typical PMMA characteristic peaks appear at 771, 841, 986 and 1058 cm^{-1}. These aforementioned peaks have become slightly enhanced in the nfPMMA-ND sample especially in the range 2840–2994 cm^{-1} due to the overlap of ND peaks in this region with those of PMMA indicating the presence of ND onto the surface of PMMA. Additionally, the disappearance of O–H vibration in the nfPMMA-ND sample indicates the involvement of this group in the immobilization of ND onto nfPMMA surface. Comparing the spectra of nfPMMA-ND with those obtained for the nfPMMA-ND–HRP sample, a clear evidence of enzyme immobilization could be observed. Typical HRP amide I and amide II bands are appearing at 1631 cm^{-1} due to C=O and 1552 cm^{-1} due to the combination of N–H bending and C–N stretching [19], indicating the success of HRP immobilization onto nfPMMA-ND. Additional confirmation could easily be observed at 3184 cm^{-1} for $–NH_2$ overlapped with –OH groups and the clear C–O–C glycosidic linkage that appears at 1038–1053 cm^{-1}. The appearance of such a glycosidic bond indicates the presence of HRP as it is a glycoprotein type enzyme [20].

The *in situ* electrospining of nanofibres in general and PMMA especially with ND is reported for the first time. PMMA with different contents of ND were then electro-spun at common condition. It can be seen that the nanofibers gradually change from smooth to rough and then harshly (Figure 2(a–d)). Typical FESEM images are also shown in (Figure 3(e,f)). It can be seen that the nfPMMA-ND-HRP nanofibers are relatively smooth surface becomes rough and even appears protruding parts with the increase of ND-HRP. It demonstrates that ND is incompletely embedded in the PMMA/ND nanofibers. Small beads start to emerge when the loading of ND is increased to 10 wt%, which are taken as the evidence to show the partial assembling of ND into polymer nanofibers [21–23]. Additionally, TEM images (Figure 4(a–c)) of nfPMMA-ND-HRP at different scales reveal clearly the apparent surface roughness and the coating materials. HRP is appearing as a white aggregate in a similar manner with previous reports [24,25]. Also, ND appears as grains with small sizes up to 10 nm, which is the typical TEM of detonation ND [26,27].

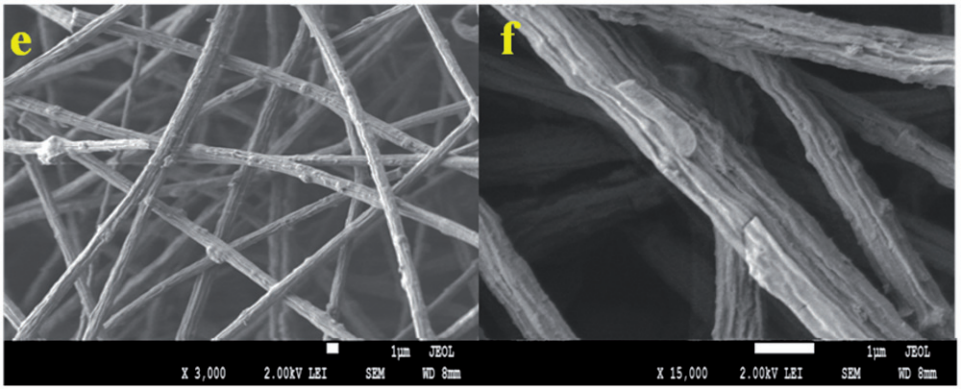

Figure 3. SEM Images of (e) nfPMMA-ND-HRP low magnification (f) nfPMMA-ND-HRP high magnification.

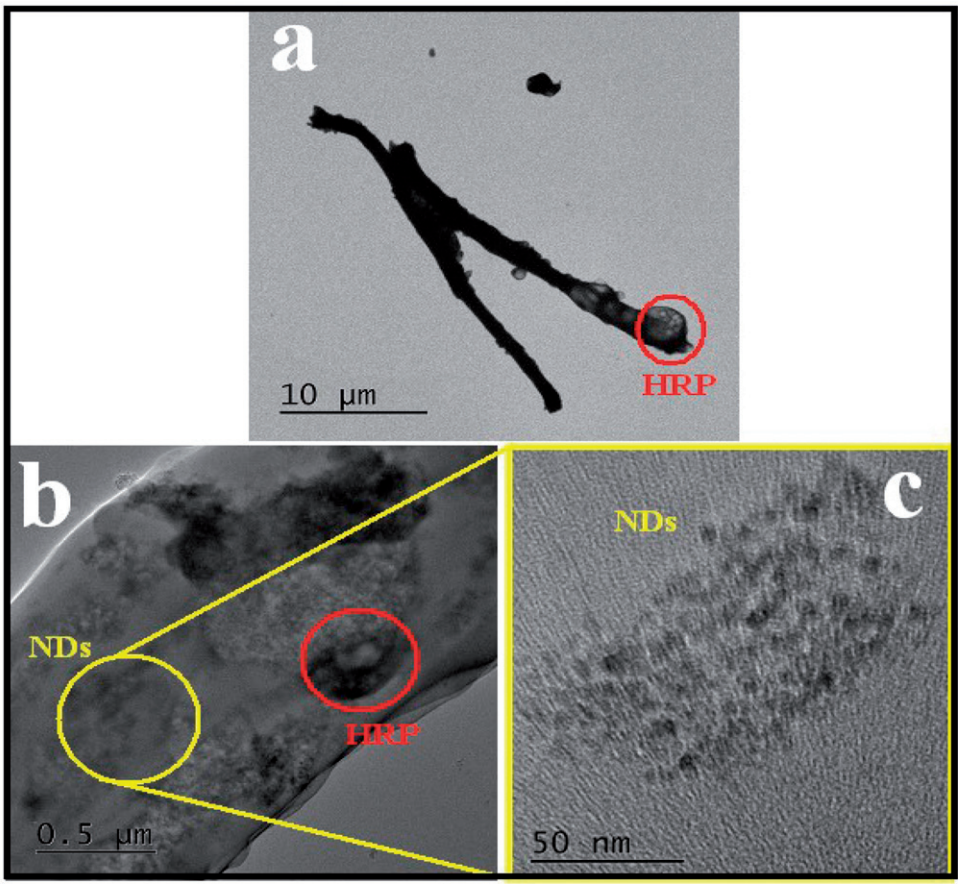

Figure 4. TEM Images of (a) nfPMMA-ND-HRP with 10 μm scale (b) nfPMMA-ND-HRP 0.5 with μm scale (c) nfPMMA-ND-HRP with 50 nm scale.

Due to the high cost of soluble enzymes, the immobilized enzymes should be reused several times. The activity loss during repeated use might be due to the inhibition of the enzyme due to its leaching during the repeated washing, a long time contact with high concentrations of H_2O_2 and damage of the supports [28,29]. Figure 5 shows the number of 10 reuses of the immobilized HRP, keeping the initial enzyme activity at 60%, indicating that the immobilized HRP has appropriate stability and can be reused. Similar results of reusability were detected in several studies [30,31].

Generally, it is possible to change the optimal pH, temperature and kinetic parameters for an immobilized enzyme as a result of the immobilized method, support structure and conformation change of enzyme after bounded, making the catalytic site more or less accessible to the substrate [32,33]. The effect of pH on activity of soluble HRP and immobilized HRP was evaluated by incubating these preparations in the buffers of varying pH values ranged from 4.0 to 9.0 (Figure 6). The pH was shifted from 7.0 for soluble HRP to 7.5 for the immobilized enzyme. Similar results were reported, where the pH was shifted from 7.0 for soluble gourd peroxidase to 7.5 for the immobilized enzyme [24]. On the contrary, the immobilized gourd peroxidase preparations showed the same pH optima as their soluble counterpart, pH 5.0 [34].

The soluble HRP had an optimum temperature of 30 °C, whereas this temperature was shifted to 40 °C for the immobilized enzyme (Figure 7). The immobilized HRP exhibited 36% of its activity at 60 °C, while the soluble enzyme retained 12%

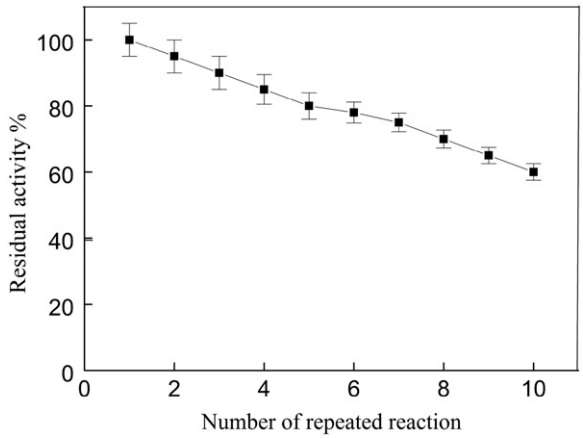

Figure 5. Reuse of immobilized HRP. Each point represents the mean of three experiments ± SE.

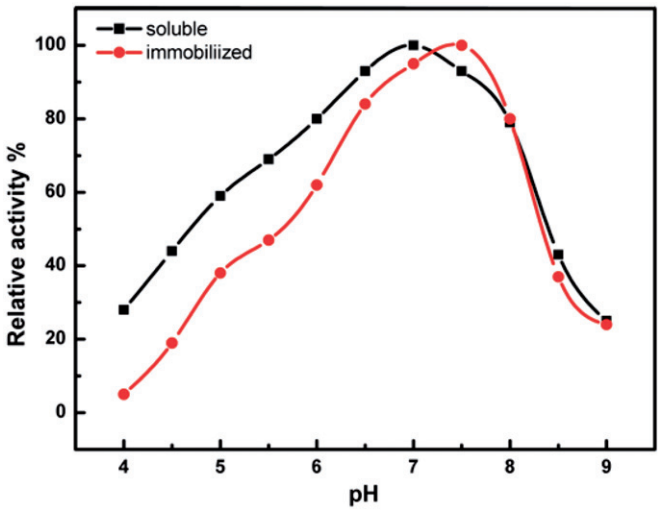

Figure 6. Optimum pH of soluble and immobilized HRP. The enzyme activity was measured at different pH's ranging from 4.0 to 9.0. Each point represents the average of two experiments.

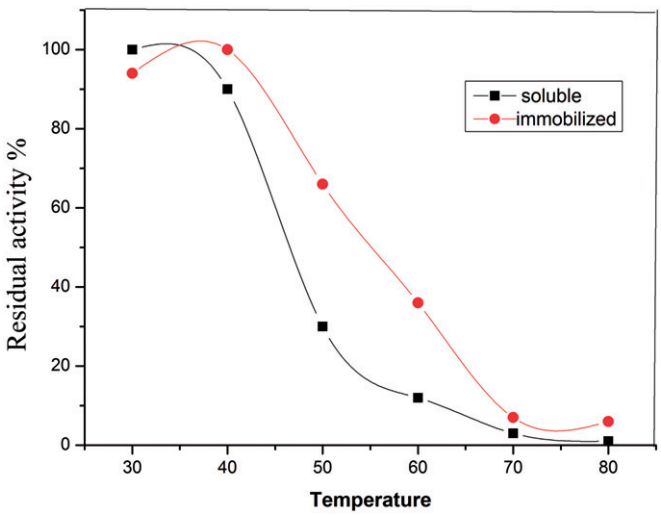

Figure 7. Optimum temperature of soluble and immobilized HRP. The enzyme activity was measured at different temperatures ranging from 30 to 80 C. Each point represents the average of two experiments.

of its activity at the same temperature. This indicated that the immobilized peroxidase resisted denaturation due to temperature rise. Several papers reported the same temperature

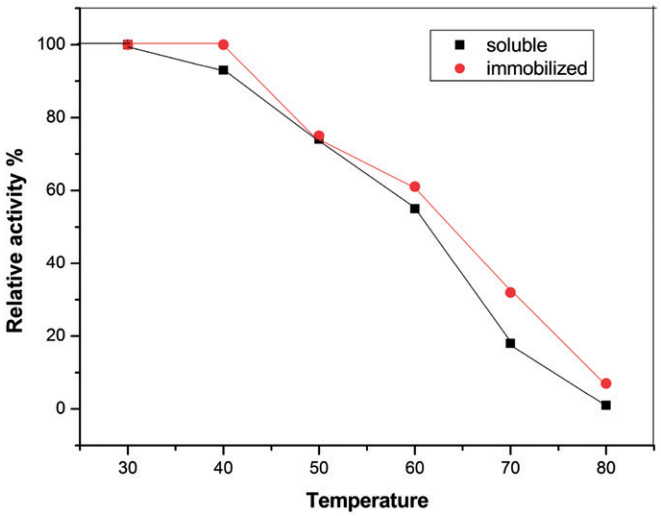

Figure 8. Effect of temperature on the thermal stability ofsoluble HRP and immobilized HRP. Each point represents the average of two experiments.

Table 2. The substrate specificity of soluble HRP and immobilized HRP.

	Relative activity %	
Substrate	Soluble HRP	Immobilized HRP
Guaiacol	100	100
o-Phenylenediamine	69	312
o-Dianisidine	34	605
p-Aminoantipyrine	25	36
Pyrogallol	17	40
ABTS	2	9

Each value represents the mean of two experiments.

optima (30, 40 °C) for the soluble and the immobilized peroxidase preparations, respectively [24,34,35]. The higher temperature initiated unfolding of protein molecules which take place more in the soluble enzyme as compared to the immobilized enzyme [36]. The thermal stability of the soluble HRP and the immobilized HRP is shown in Figure 8. The results showed that the soluble HRP and the immobilized HRP were thermally stable up to 30 °C and 40 °C, and retained 55% and 61% of its activity at 60 °C, respectively. These results showed that the immobilized HRP is more thermally stable than soluble HRP at all temperature tested from 30 to 80 C. Improvement in the thermal stability of the immobilized enzyme may come from a multipoint complexation of enzyme with the support [37]. The thermal stability of the immobilized enzymes compared to soluble enzymes is very important for the industrial applications, where high temperatures are needed to catalyze the reaction rate [38].

Table 2 shows the substrate specificity of soluble HRP and immobilized HRP. Various substrates such as guaiacol, *o*-phenylenediamine, *o*-dianisidine, *p*-aminoantipyrine, pyrogallol and ABTS were oxidized by immobilized HRP with higher efficiencies than those of the soluble HRP. On the contrary, Valerio et al. [39] reported that the reduction in the affinity of immobilized enzyme of the substrate compared to soluble form could be attributed to the uneven surface of the support, the high concentration of protein that was immobilized generating diffusion effects and the change of active site of enzyme after contact with the solid surface of the support. Figure 9 shows Km values of the soluble HRP and the

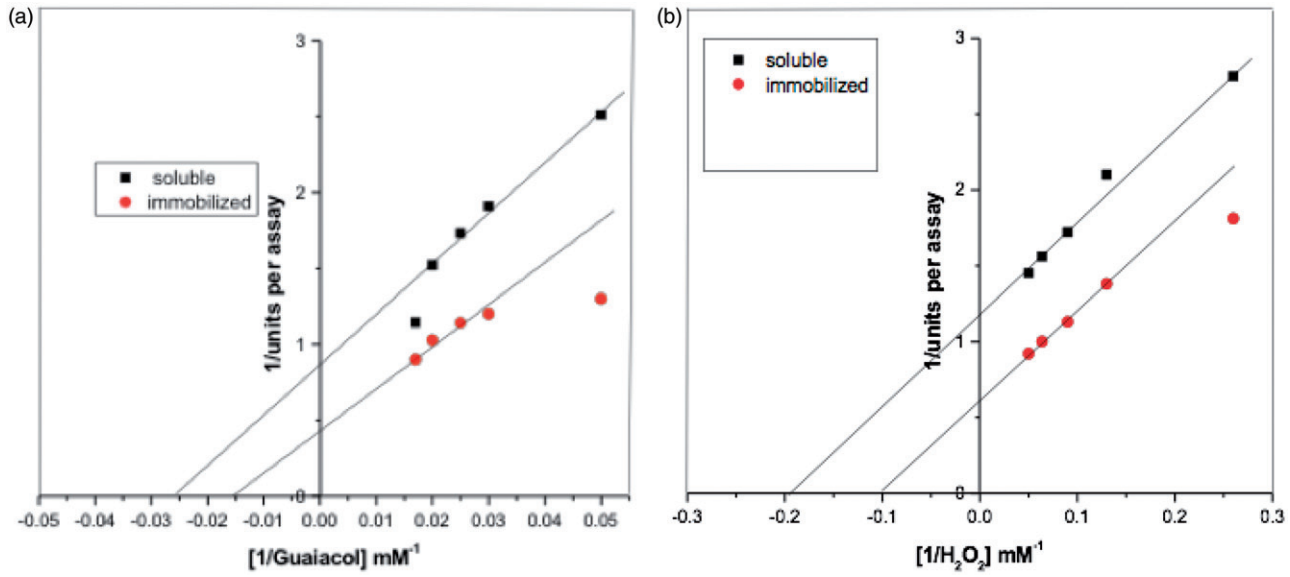

Figure 9. Lineweaver-Burk plots relating soluble HRP and immobilized HRP reaction velocity to guaiacol (a) and H_2O_2 (b) concentrations. Each point represents the average of two experiments.

Table 3. The effect of 5 mM metal ions on the activity of soluble HRP and immobilized HRP.

	Relative activity%	
Metal ion	Soluble HRP	Immobilized HRP
Control	100	100
Co^{2+}	73	97
Ca^{2+}	109	155
Pb^{2+}	75	87
Ni^{2+}	66	80
Zn^{2+}	65	83
Hg^{2+}	16	83
Cu^{2+}	89	289
Fe^{3+}	168	293
Cd^{2+}	86	92

Each value represents the mean of two experiments.

Table 4. The effect of chemical compounds on soluble HRP and immobilized HRP.

		Relative activity%	
Chemical compounds	concentration	Soluble HRP	Immobilized HRP
Control		100	100
Urea	2M	60	82
	4M	24	50
Isopropanol	5%	39	77
	10%	31	87
n-Butanol	5%	75	92
	10%	63	76
Heptane	5%	68	96
	10%	55	84

Each value represents the mean of two experiments.

immobilized HRP were 38 mM and 64 mM for guaiacol and 5.0 mM and 9.8 mM for H_2O_2, respectively. The results showed that the soluble HRP had more affinity toward guaiacol and H_2O_2 than the immobilized HRP. Several studies reported that the Km of immobilized enzyme was higher than that of soluble enzyme [25,30]. The immobilization affects the diffusion of the substrate toward the active site of the enzyme, this being reflected by a considerable increase in the apparent affinity constant (Km) when compared with that corresponding to the free enzyme [40].

Metal ions induce conformational changes in enzymes, where soluble horseradish peroxidase was remarkably inhibited by heavy metal ions [41,42]. Our results revealed that the inhibition of the immobilized HRP by metal ions was low as compared to the soluble enzyme (Table 3). The most of metal ions caused partially inhibitory effect toward soluble enzyme, while Fe^{3+} and Ca^{2+} caused great and partially activation effect, respectively. Fe^{3+}, Cu^{2+} and Ca^{2+} caused activation effects toward immobilized HRP. The other metals tested caused slightly inhibitory effects. Although Hg^{2+} caused strong inhibition of the activity of the soluble HRP, the immobilization of enzyme partially protected them from this harmful ion. The stability of the immobilized peroxidases against several metals showed that such preparations could be exploited to treat aromatic pollutants even in the presence of metal ions [43].

The protein unfolding by direct interaction of urea molecule with a peptide backbone via non-covalent interactions contributes to the maintenance of protein conformation. Generally, a high concentration of urea caused unfolding of protein followed by a decrease in the activity of enzymes [44]. Therefore, exposure of soluble HRP and immobilized HRP with urea at 2 and 4 M for 15 min was determined as shown in Table 4. The immobilized HRP retained 82% and 50% of its activity at 2 and 4 M urea, while soluble enzyme retained 60% and 24%, respectively. This is in agreement with the resistance of chitosan horseradish peroxidase [35], Concanavalin A-pointed gourd peroxidase [36], activated wool horseradish peroxidase [25], and amidoximated acrylic polymer [24] against high concentration of urea.

In the present study, the activity of the immobilized HRP was markedly more stable when it exposed to isopropanol, butanol and heptane (5% and 10%) (Table 4). Isopropanol caused a slightly inhibitory effect on the activity of the immobilized HRP, where it caused a strong inhibition effect on the activity of the soluble HRP. Butanol and heptane caused inhibitory effect toward immobilized enzyme less

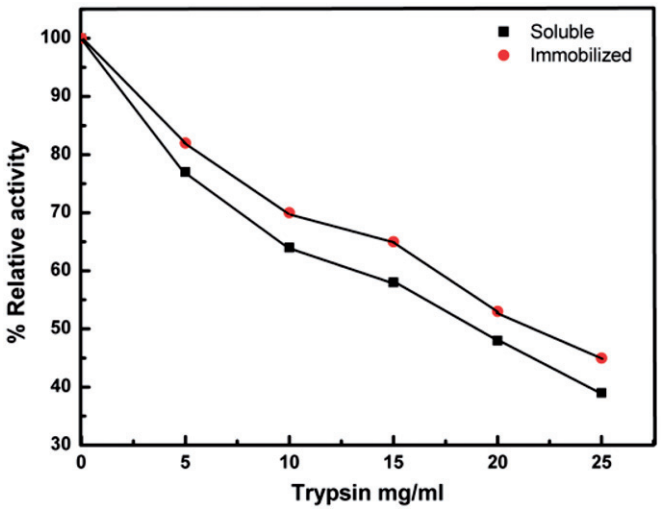

Figure 10. The effect of trypsin concentration on the activity of soluble HRP and immobilized HRP. Each point represents the average of two experiments.

than soluble enzyme. The investigation of the stability of enzymes in organic solvents is very important due to the presence of organic solvents in wastewater. It has been reported that several immobilized peroxidases exhibited more resistance to organic solvents [23,35,45].

Figure 10 shows the stability of soluble and immobilized HRP in the presence increasing the concentration of trypsin from 5 to 25 mg. The immobilized HRP exhibited high resistance to proteolysis by trypsin than that of soluble enzyme. The similar results reported that the soluble peroxidases were more inactivated in presence of trypsin than the immobilized enzymes [44,45].

Conclusion

The nfPMMA-ND nanofibers were prepared and characterized for immobilization of HRP. HRP immobilized on nfPMMA-ND showed a very high yield of enzyme immobilization and good reusability. The immobilized HRP was more stable towards the denaturation induced by pH, heat, metal ions, urea, proteolytic enzyme and water-miscible organic solvent. The observed results appeared that the immobilized HRP could be employed in several applications such as biosensor, biomedical and bioremediation.

Disclosure statement

No potential conflict of interest was reported by the authors.

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