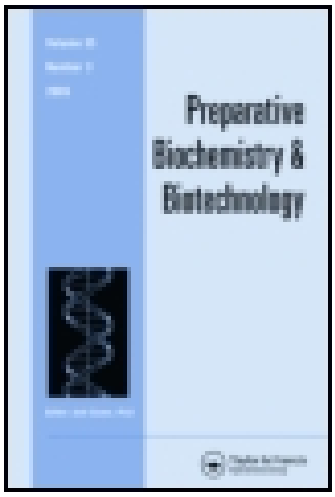

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Immobilization of Horseradish Peroxidase on β-cyclodextrin Capped Silver Nanoparticles: Its Future Aspects in Biosensor Application

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Immobilization of horseradish peroxidase on β-cyclodextrin capped silver nanoparticles: Its future aspects in biosensor application

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Abstract

The present study aimed to work out a simple and a high yield procedure for the immobilization of horseradish peroxidase on silver nanoparticle. UV-Vis, Fourier transform infrared and transmission electron microscopy was used to characterize silver nanoparticles. Horseradish peroxidase was immobilized on β-cyclodextrin capped silver nanoparticle via glutaraldehyde crosslinking. Single cell gel electrophoresis (Comet assay) was also performed to confirm the genotoxicity of silver nanoparticles. To decrease toxicity, silver nanoparticles was capped with β-cyclodextrin. A comparative stability study of soluble and immobilized enzyme preparations was investigated against pH, temperature and chaotropic agent; urea. The results showed that the cross-linked peroxidase was significantly more stable as compared to their soluble counterpart. The immobilized enzyme exhibited stable enzyme activities after repetitive uses.

KEYWORDS: β-cyclodextrin; horseradish peroxidase; immobilization; silver nanoparticle; stability

INTRODUCTION

Nanosized materials have been predominantly employed in various areas including catalysis, mechanics, optics, magnetics, energetics and biomedical sciences. An important application of nanoparticle is the development of biosensors and nanoscale electronic devices. Nanoparticles exhibited excellent features like high surface-to-volume ratio, thermal and chemical stability and biocompatibility thus provide stable environment for the enzyme immobilization. Additionally, nanoparticle enzyme complex possess less stearic hindrance and diffusion limitations as well as high enzyme loadings.[1]

Immobilization of proteins on solid support strongly depends on the nature of protein and surface geometry and physicochemical characteristics of support. Various specific and nonspecific interactions such as hydrogen bonding, electrostatic and hydrophobic interactions takes part in binding of protein with nanoparticles which may affect the structure and stability of proteins.[2,3] Nanoparticles coated with natural or synthetic polymer like cellulose, polyaniline, gelatin, chitosan, montmorillonite and cyclodextrin form core-shell activated biocompatible support for the immobilization of biomolecules.[4-8] Cyclodextrins (CD) are cyclic oligosaccharides containing six, seven or eight glucose units (α, β and γ-CD, respectively) linked with α-(1,4) bonds. It has toroidal shape with interior hydrophobic receptive cavity and hydrophilic exterior. The hydrophobic cavity is capable in forming inclusion complexes both in solution and in solid state with different hydrophobic compounds like numerous amino acid residues present at surface of proteins.[9,10] Therefore, multipoint interactions of enzyme with CD, increase their stability and favor immobilization.

Hydrogen peroxide is an important byproduct in horseradish peroxidase (HRP) catalyzed reactions acts as a signal transducer molecule in food, pharmaceutical, clinical and environmental assays. Therefore, the detection of H_2O_2 in samples is linked with performance of HRP biosensors.[11] The present study is focused on the synthesis of β-cyclodextrin (βCD) capped nanoparticles (AgNPs) for stable and economic immobilization of HRP. Toxicity of AgNPs was reduced by capping with βCD. A comparative stability study of soluble and immobilized enzyme was carried out against various physical and chemical denaturants.

MATERIALS AND METHODS

Silver nitrate ($AgNO_3$), ethanol, sodium borohydride, trisodium citrate dihydrate, dimethylformamide, bovine serum albumin, *o*-dianisidine HCl and HRP were obtained from Sigma Aldrich. Other chemicals and reagents employed were of analytical grade.

Preparation Of B-CD Capped Silver Nanoparticle

Aqueous solution of $AgNO_3$ (0.01 M, 1 ml) and trisodium citrate dihydrate (0.03 M, 1 ml) were added into an ultrapure water (97 ml) and mixed slowly. One ml of sodium borohydride (1.79 mg/ml) was added drop wise into $AgNO_3$ solution with continuous stirring. After 20 min of reaction, transparent colorless solution was converted into characteristic pale yellow color indicated the formation of silver nanoparticles. βCD and AgNPs (1:2 ratios) were mixed for 24 h with continuous stirring. Formed precipitate was collected by centrifugation and washed with dimethylformamide (DMF) (4 x 50 ml) to

remove free thiolated βCD. The pellet was further washed (4 x 50 ml) with ethanol/water (90:10 v/v), collected by centrifugation and dried at 60 °C under vacuum. The resulting powder was suspended in distilled water to make it 9.85 mg/ml.[12]

Characterization

Morphology and size of AgNPs was investigated by transmission electron microscope (JEOL, 2000FX, Japan) at 200 kV accelerating voltage. UV-vis absorption spectrum was recorded with Shimadzu dual beam spectrometer (Shimadzu, Kyoto, Japan) in the wavelength range of 200-800 nm. FT-IR spectral studies were performed using KBr pelleting method with Perkin Elmer System 2000 instrument in the range of 500-4000 cm^{-1}.

Comet Assay

The Comet assay was performed according to Singh et al with minor modification.[13] Briefly, AgNPs and βCD capped AgNPs treated lymphocytes were mixed with 50 µl of 1% low melting point agarose (LMA) at 37 °C. These cells were transferred onto fully frosted slides which already coated with 1.0% normal melting point agarose (NMA). The slides were covered and kept at 4 °C for solidification of agarose. Slides were immersed in the lysing solution (2.5 mM NaCl, 1% Triton X-100, 10% DMSO, 10 mM EDTA, 10% sodium lauroyl sarcosinate and 100 mM Tris, pH 10) for 1 hour and then placed into unwinding buffer (30 mM NaOH and 1.0 mM EDTA, pH >13) for 20 min. The electrophoresis was performed, stained with ethidium bromide and imaged with Olympus fluorescent microscope at 200X magnification.

Immobilization Of HRP With Bcd Capped Agnps

Prepared βCD capped AgNPs were mixed in sodium phosphate buffer pH, 8 at 4 ^{o}C having different units of HRP to achieve best nanoparticles peroxidase ratio for immobilization. The mixture was homogenized for 20 min at 4 ^{o}C. The resulting solution was then centrifuged at 2991xg for 15 min and filtrate freeze-dried for 24 hours. The immobilized preparation was cross-linked using 0.5% glutaraldehyde for 1 h as described by Karim and Adnan.[14] Finally crosslinked preparation was suspended in 100 mM sodium phosphate buffer, pH 8 and stored at 4 ^{o}C for further use.

Measurement Of Peroxidase Activity

Peroxidase activity was determined by the change in optical density (A_{460} nm) at 40 °C through measuring initial rate of *o*-dianisidine HCl (18 mM) oxidation by H_2O_2 (6 mM). One unit (1 U) of peroxidase activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µM of *o*-dianisidine HCl per min at 37 °C.

Effect Of Ph On Soluble And Agnps-HRP

Equal amounts of soluble and crosslinked AgNPs-HRP (2 U each) were taken to determine the activity of peroxidase in the buffers of different pH. The buffers used were glycine-HCl (pH 2 and 3), sodium acetate (pH 4 and 5), sodium phosphate (pH 6 and 7) and Tris-HCl (pH 8-10). The activity at pH-optimum was considered as control (100%) for the calculation of percent activity at remaining pH.

Effect Of Temperature On Soluble And Agnps-HRP

The activity of soluble and crosslinked AgNPs-HRP (2 U) was determined at various temperatures (20-80 °C) in sodium phosphate buffer, pH 8. The activity at temperature-optimum was considered as control (100%) for the calculation of percent activity at other temperatures.

Effect Of Urea On Soluble And Agnps-HRP

Soluble and AgNPs-HRP (2 U) were incubated with 4 M urea for varying times periods in 100 mM sodium phosphate buffer, pH 8 at 40 °C. The activities of both the preparations were determined at indicated time intervals. The activity of enzyme without incubation with urea was taken as control (100%) for the calculation of remaining percent activity.

Reusability Of Agnps-HRP

Immobilized enzyme preparation was taken in triplicates for assaying peroxidase activity to 5 successive days. After each assay enzyme preparations were taken out, washed and stored in 100 mM sodium phosphate buffer, pH 8 for overnight at 4 °C. The activity determined for the first day was considered as control (100%) for the calculation of remaining percent activity after each use.

Statistical Analysis

Each value represents the mean of three independent experiments performed in duplicates with average deviations <5%. Data expressed in various studies were plotted using Sigma Plot-10 and Microsoft Excel 2003. *P*-values <0.05 were considered statically significant.

RESULTS AND DISCUSSION

Characterization Of Silver Nanoparticles

AgNPs were prepared by sodium borohydride reduction of silver nitrate with a citrate stabilizer. The method ensured that the nanoparticles provided a strong surface plasmon band that could be used in colorimetric assay. AgNPs was surrounded by negatively charged citrate ions which provided enough electrostatic repulsion to overcome the attractive hydrophobic and van der Waals forces. Therefore, citrate ions stabilized AgNPs and make dispersed in an aqueous solution. [15]

Optical properties of metallic nanoparticle in aqueous suspension are closely associated with their shape.[16] Prepared AgNPs showed the maximum absorption peak at 400 nm (Figure 1). The UV-vis absorption spectra of AgNPs were monitored up to 24 hours by periodic sampling of aliquots, exhibited unified patterns with an increasing NPs concentration in the extract. This result coincides with the previously published data.[17] Figure 2 represents transmission electron micrograph of AgNPs. This is in concordance with the shift in ultraviolet spectrum of AgNPs. The average size of AgNPs was ~10 nm. Synthesized βCD and βCD modified AgNPs was monitored by infrared spectrum which demonstrated successful formation of βCD-modified AgNPs (Figure 3). A stretching vibration peak of S-H for βCD was observed at 2569 cm^{-1} which is not present in βCD

modified AgNPs. This showed the successful formation of βCD and anchoring β-SH-CD on AgNPs.[18]

Assessment Of DNA Breakage

AgNPs as well as βCD capped AgNPs were tested for DNA breakage in intact lymphocytes by Comet assay (Figure 4). Photographs of Comet exhibited no breakage in case of βCD capped AgNPs treated DNA (Figure a T1) while it conform toxicity in AgNPs treated cells (Figure a T2). Histogram of Comet demonstrated the percentage DNA breakage in tail because the length of tail is directly proportional to DNA damage (Figure 4b). The result showed that there was significant increase in tail length of DNA treated with AgNPs (Figure b T1) while no remarkable tail length arose in βCD capped AgNPs treated DNA. Flower et al reported that the AgNPs even at 5 min of treatment induced DNA damage in human peripheral blood cells.[19]

Preparation Of Bcd-HRP Complex

βCD has been exploited for the immobilization of peroxidase on βCD capped AgNPs. Enzyme was maximally adsorbed at pH 8.0 with retention of 92% enzyme activity (Table 1). Crosslinking of adsorbed HRP by 0.5% glutaraldehyde resulted in a loss of 1% activity (Table 1). Immobilization of enzyme on macro support has several demerits such as low activity and stability, expensive immobilized preparation and leaching of enzymes from support while immobilization on nanosupport takes numerous advantages like higher enzyme activity and stability, low mass transfer resistance of substrate and products and superior loading capacity.[20,21]

Ph Activity Profiles For Soluble And Agnps-HRP Preparation

Figure 5 demonstrates pH-activity profiles for soluble and AgNPs-HRP preparation over a range of pH, 2.0-10.0. AgNPs-HRP preparation showed no change in pH-optimum (pH 8.0) but retained significantly higher enzyme activity both in acidic and alkaline region as compared to free enzyme. Immobilized HRP showed 84% activity at pH 10.0 whereas free enzyme retained 65% under same experimental conditions. Furthermore, in acidic region (pH 2.0) AgNPs-HRP exhibited 59% activity while the activity of soluble HRP was only 36%. The activity of enzyme may change after immobilization might be due to pH change in local environment by properties of matrix and in the domain of immobilized enzyme molecule.[22] The broadening of pH activity profile for immobilized peroxidase was also observed in a number of prior studies.[23,24]

TEMPERATURE ACTIVITY PROFILES OF SOLUBLE AND AGNPS-HRP

The effect of temperature on the activities of free and AgNPs bound HRP were evaluated in the range of 20-80 °C (Figure 6). Result showed that both the preparations exhibited temperature optima at 40 ^{o}C. However, AgNPs bound HRP retained more catalytic activity at higher temperatures. It was noticed that AgNPs bound HRP retained 40% activity at 80 °C, whereas free enzyme habited 20% activity at same temperature. The elevated activities of immobilized HRP at higher temperature is due to conformational limitation of free enzyme movement as a result of electrostatic interaction and hydrogen bonding between the enzyme and support which decrease the probability of thermal denaturation. Furthermore, active site of immobilized enzyme exposed more

appropriately at higher temperature for the substrate thereby increased enzyme activity.[25,26]

Urea Mediated Denaturation Of Soluble And Agnps-HRP

Nanoparticles bound HRP was more resistant to inactivation induced by urea as compared to its soluble counterpart. Exposure of soluble enzyme with 4 M urea for 2 h resulted in the loss of 70% activity whereas the immobilized enzyme retained more than 80% of the initial enzyme activity (Figure 7). Urea is a strong denaturant however, action mechanism of urea on protein structure has not yet been completely understood.[27] Several earlier findings have suggested that protein might be unfolded by direct interaction of urea molecule with a peptide backbone via hydrogen bonding and/or hydrophobic interaction which contribute in the maintenance of protein conformation.[28,29] Ikemoto et al reported that urea interacts directly with protein in the denaturation process to pull away the water surrounding the protein. However, in case of immobilized enzyme, it could not replace water in the confined space.[30]

Reusability Of Agnps-HRP

Reusability of immobilized HRP has been shown in Figure 8. After 5th repeated use, AgNPs bound HRP retained 97% of the original activity. Enzyme reuse provides a number of advantages which is an essential prerequisite for establishing an economically viable enzyme-catalyzed process. The loss of activity during repeated uses is might be due to product inhibition or leaching of enzyme from the support.

CONCLUSION

The results presented in the present work showed that AgNPs bound HRP was more stable as compared to soluble against various denaturants and retained higher activities than its soluble counterparts. Therefore, such immobilized enzyme preparation can be used for the preparation of biosensors for the detection/monitoring of pollutants present in wastewater. These immobilized complex used in a reactor would not affect the flow rate of the column. In view of these advantages we can suggest that AgNPs bound HRP would be suitable for the treatment of huge volume of effluents/aromatic pollutants present in wastewater.

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Table 1. Immobilization of HRP on AgNPs

Enzyme Loaded (X) (U)	Enzyme activity in washes (Y) (U)		Activity bound to AgNPs (U)		(%) Activity yield (B/A×100)	(%) Activity after crosslinking
		Theoretical (X-Y=A)	Actual (B)	Effectiveness factor (B/A) = (η)		
3400	491	2909	2681	00.92	92%	91%

Figure 1 UV-vis absorption spectrum of AgNPs. UV-vis absorption spectrum of Ag-NPs was monitored with Shimadzu dual beam spectrometer (Shimadzu, Kyoto, Japan) operated at resolution of 2.0 nm. The spectrum was recorded in the wavelength range of 200 to 800 nm.

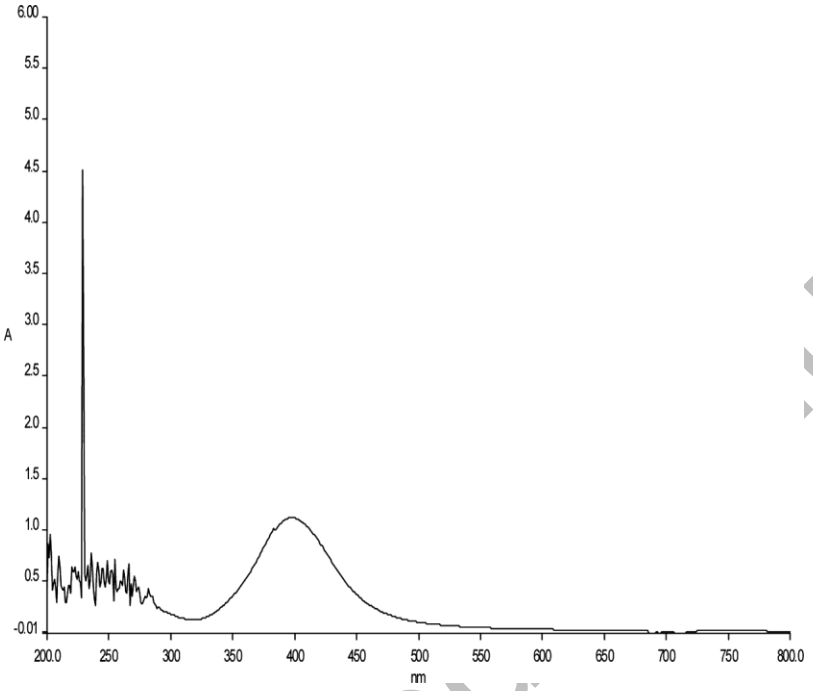

Figure 2 Transmission electron micrograph of AgNPs. TEM image of AgNPs was investigated by transmission electron microscope (JEOL, 2000FX, Japan) at 200 kV accelerating voltage.

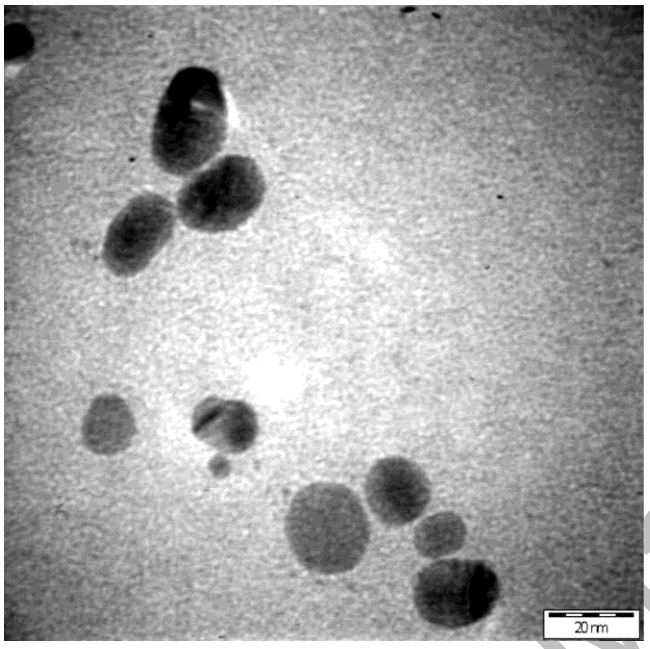

Figure 3 FT-IR spectra of βCD and βCD capped AgNPs. FT-IR spectra of βCD (a) and βCD capped AgNPs (b) were monitored with Perkin Elmer System 2000 instrument in the range of 500-4000 cm^{-1}.

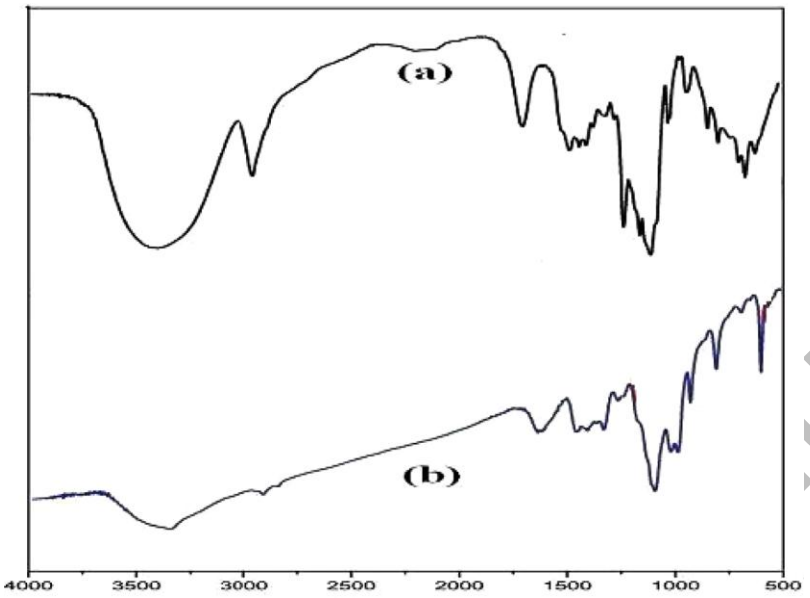

Figure 4 Comet assay of AgNPs and βCD capped AgNPs. Nuclear DNA breakage was analyzed by Comet assay as given in the text. (A) The microscopic images has been shown for AgNPs treated DNA (T1) and βCD capped AgNPs treated DNA (T2). (B) Comet assay histogram shown AgNPs treated DNA (T1) and βCD capped AgNPs treated DNA (T2).

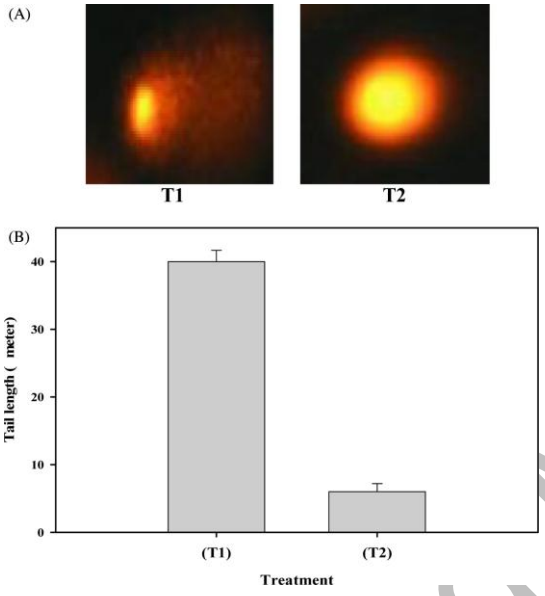

Figure 5 pH-activity profiles for soluble and AgNPs-HRP. The enzyme activity for soluble and AgNPs-HRP was measured in the buffers of various pH. Buffers used were glycine-HCl (pH 2 and 3), sodium acetate (pH 4 and 5), sodium phosphate (pH 6 and 7) and Tris-HCl (pH 8-10). The activity at pH-optimum was considered as control (100%) for the calculation of percent activity at remaining pH.

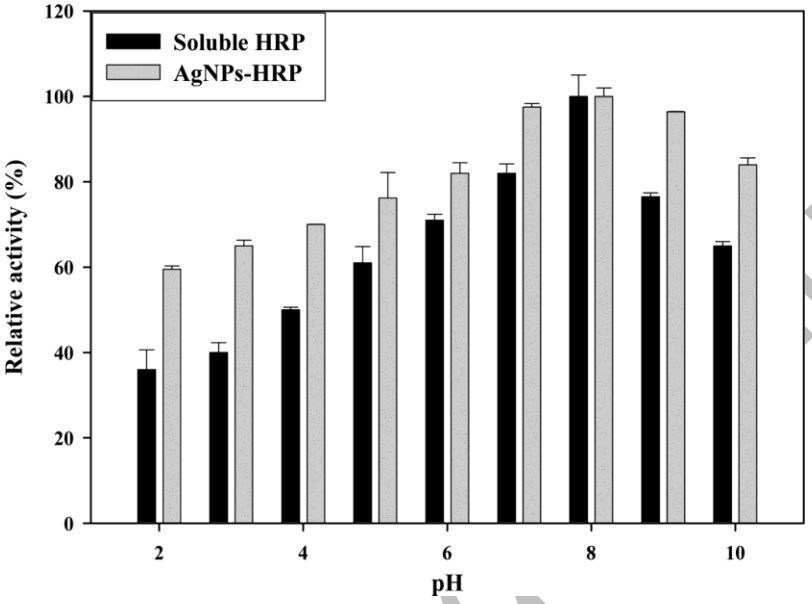

Figure 6 Temperature-activity profiles for soluble and AgNPs-HRP. The enzyme activity of soluble and AgNPs-HRP was measured at various temperatures (40-80 ^{o}C). The activity at temperature-optimum was considered as control (100%) for the calculation of percent activity at other temperatures

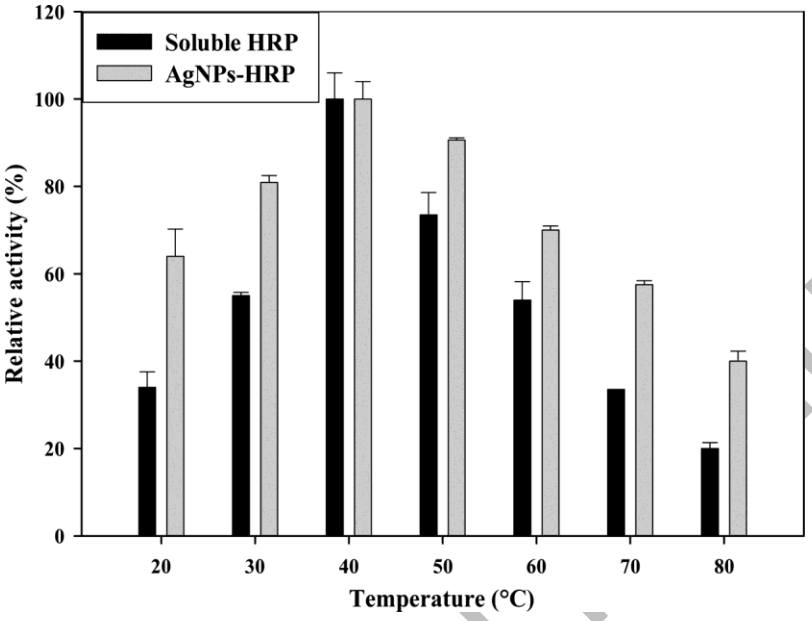

Figure 7 Effect of urea on soluble and AgNPs-HRP. Soluble and AgNPs-HRP was incubated with 4 M urea in a 100 mM sodium phosphate buffer, pH 8 at 40 °C. Enzyme activity was determined at different time intervals. Untreated samples were considered as controls (100%) for the calculation of remaining percent activity.

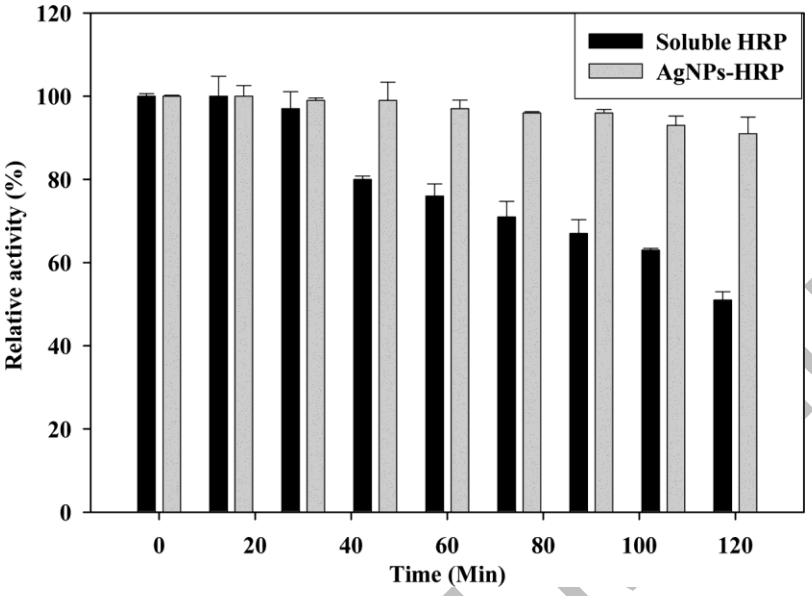

Figure 8 Reusability of AgNPs-HRP. The reusability of AgNPs-HRP was monitored for 5 successive days. The activity determined on the first day was taken as control (100%) for the calculation of remaining percent activity after each use.