Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
*J. Serb. Chem. Soc. 78* (7) 921–931 (2013)
*JSCS–4470*
UDC 633.846+541.459:620.28:577.15:544.478–035.2
*Original scientific paper*

# Magnetic labelled horseradish peroxidase–polymer nanoparticles: a recyclable nanobiocatalyst

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(Received 30 September, revised 26 November 2012)

*Abstract*: In this study, the reusability and process stability of nano-reengineered horseradish peroxidase was investigated in a fluorescence-based sensing system for hydrogen peroxide determination as a model application. To this end, dendron macromolecules were attached to the enzyme surface through bio-conjugation techniques. The resulting enzyme–polymer nanoparticles, with an average size of 14(±2) nm, showed significant life time and thermal stability. For enzyme recovery and reusability purposes, the enzyme–polymer nanoparticles were labelled with magnetic nanoparticles with a labelling yield of 90 %. These labelled enzyme molecules showed significant process stability, *i.e.*, up to 7 recycling period in a model sensing system. A linear calibration curve was obtained over a hydrogen peroxide concentrations range from $5\times10^{-8}$ to $1\times10^{-5}$ mol $L^{-1}$, with a detection limit of $1.3\times10^{-9}$ mol $L^{-1}$ for the sensing system under the optimal conditions.

*Keywords*: horseradish peroxidase; nano-reengineered enzyme; magnetic nanoparticles; recyclable biocatalyst.

## INTRODUCTION

Enzymes, nanometric biocatalysts that play a key role in biochemical reactions of living cells and bio-organisms, offer various applications, including biosensing, bioremediation and chemical synthesis[1–3] due to their specificity.[4,5] Nevertheless, the relatively short lifetime of enzymes and their instability in harsh environments (elevated temperature and organic media) limit their applications.[4] Enzyme stabilization can provide several advantages including: 1) decrease in the amount of enzyme required, 2) life time prolongation and 3) enhancement in the potential for enzyme reuse.[2,6]

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doi: 10.2298/JSC120930133K

There have been several approaches to improve enzyme stability that are mainly categorized into enzyme immobilization (entrapment on solid surfaces or within porous materials,[7,8] trapping in gels,[9] polymers[10] or composite materials[11]), surface modification, protein engineering and reaction medium engineering.[12–14]

The economics of a bio-catalytic process can be enhanced by enzyme reuse. One of the most attractive methods of separating the enzyme molecules from the reaction solution is utilizing magnetic nanoparticles–enzyme conjugates.[15,16] The magnetic nanoparticles can attach to the enzyme surface through conjugation methods or they can play the role of a support for enzyme immobilization. Suspended super-paramagnetic particles in solution can be removed from a reaction mixture using an external magnet, but they do not agglomerate after removal of the external magnetic field.[17]

In the past years, magnetic nanoparticles have been widely used as biomolecule-carriers for the immobilization of various enzymes.[18–21]

However, the activity and stability of enzyme immobilized on these magnetic nanoparticles will greatly depend on the environmental factors, such as pH, temperature and organic solvent.[17] To improve this limitation, Yang and co-workers fabricated magnetic single enzyme nanoparticles through encapsulation of each single glucose oxidase molecule in a thin composite layer of magnetic nanoparticles and polymer.[17] In a previous work, a simple method was proposed for the fabrication of enzyme–polymer nanoparticles using dendritic polymers, which improved the enzyme stability through bio-conjugation of polyester dendron macromolecules to the primary amino groups on the enzyme surface. The approach represents a novel way of modifying and stabilizing enzymes as the fabricated horseradish peroxidase–polymer nanoparticles exhibited significant life-time stability up to 70 days, whereas free horseradish peroxidase (HRP) lost its activity after several days.[22] These enzyme–polymer nanoparticles could readily be labelled with magnetic nanoparticles for further enzyme recovery and reused by employing an external magnetic field for enzyme separation from the reaction mixture.

The present study was aimed at evaluating the process stability and reusability of magnetic-labelled dendritic modified enzymes. As a model application of HRP, a hydrogen peroxide ($H_2O_2$) sensing system was designed based on the generation of a fluorescent agent from the reaction of homovanillic acid (HVA) and $H_2O_2$ in the presence of the stabilized HRP.[23,24] This simple and model enzyme-based process was used to investigate the stability and reusability of the labelled modified enzyme. In addition, the sensing conditions were optimized for this nano-reengineered HRP and also the linear range and detection limit of the sensing system were examined. Furthermore, as a real test, the $H_2O_2$ content of some excipient samples was assessed. Excipients are the inactive part of drug

formulations that have some roles, such as binder, filler or dispersing agent, for the active drugs. Drug chemical stability could be significantly decreased by impurities present in formulation excipients. Hydrogen peroxide is one of the impurities that could oxidize drugs. For example, the presence of residual $H_2O_2$ in poly(vinylpyrrolidone) (PVP) as a pharmaceutical excipient is reported to be the main agent in drug oxidation and deactivation.[25,26]

## EXPERIMENTAL

### *Materials*

Horseradish peroxidase (HRP), 3,3′,5,5′-tetramethylbenzidine (TMB), *N*-hydroxysulphosuccinimide (Sulpho-NHS), 2-(4-morpholino)ethanesulphonic acid (MES), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) and ethylenediamine (EDA) were purchased from Merck (Germany). Polyester-32-hydroxyl-1-carboxyl bis-MPA Dendron (Generation 5, $M_w$ 3617 g $mol^{-1}$) and HVA were purchased from Sigma–Aldrich (USA). A solution of $Fe_3O_4$ nanoparticles (7 vol. %) was purchased from PlasmaChem (Germany). All other chemicals were of analytical grade and used without further purification. De-ionised (DI) water (resistivity of 18 MΩ cm) was obtained from a Millipore Milli-Q Water System (Millipore Inc.), and was used for rinsing and for makeup of all aqueous solutions.

### *Methods*

*Enzyme–polymer bio-conjugation*. As reported in a previous work, a dendritic polymer with a carboxylic group core was conjugated to amine group residues of HRP for stability enhancement.[22] Briefly, the dendron was dissolved in MES buffer (pH 6.0, 0.05 M) at a concentration of 1 mg $mL^{-1}$. EDC and Sulpho-NHS were added to reach the final concentrations of 2 mM for EDC and 5 mM for Sulpho-NHS. They were mixed and reacted for 15 min at room temperature. Then, an HRP solution (1 mg $mL^{-1}$ in 0.1 M phosphate buffer of pH 7.5) was added and mixed for at least 2 h and subsequently the fabricated HRP–dendron nanoparticles were purified.

*Labelling of HRP–dendron nanoparticles with magnetic nanoparticles*. The purchased magnetic nanoparticles (MNPs) had oleic acid molecules on their surface as the stabilizer. Ethylenediamine was used for the conjugation of the surface acid groups of the MNPs to the carboxyl groups on the surface of the HRP–polymer nanoparticles. At first, 200 μL of a MNPs solution (7 vol. %) was diluted with 5 mL of PBS buffer (0.10 M). Then, it was activated with EDC and Sulpho-NHS solutions with final concentrations of 2 mM and 5 mM, respectively. Then, 5 mL of HRP–polymer nanoparticles solution was also activated with EDC and Sulpho-NHS. After stirring each mixture for 15 min, 100 μL of ethylenediamine and the enzyme nanoparticles mixture were simultaneously added to the first mixture under mechanical stirring conditions and mixed for 1 h. Then, a strong magnet was used for the separation of MNPs from the mixture. Finally, the separated MNPs (attached to the HRP–polymer nanoparticles) were washed 3 times with DI water to remove excess reactants and then stored in the refrigerator at 4 °C. The HRP concentration was evaluated using the Lowry procedure as modified by Peterson[27] for determination of the HRP labelling yield.

### *$H_2O_2$ fluorescence-based nanobiosensor*

Homovanillic acid (HVA) was used as a fluorogenic compound for $H_2O_2$ sensing. HVA itself is not a fluorescent agent but after oxidation with $H_2O_2$ in the presence of HRP converts

to a fluorescent dimer. Therefore, it could be used as a probe in the fluorescence-based $H_2O_2$ sensors.

Reaction solution (3 mL) was obtained by mixing 2.2 mL phosphate buffer saline (PBS, 0.10 M) of pH 7.5, 0.3 mL HRP solution and 0.2 mL HVA solution. The reaction was started by addition of 0.3 mL of $H_2O_2$ solution at a pre-defined concentration (or the sample solution) to the above mixture. Then, the assay solution was mixed for 5 min at room temperature. At the end of reaction, the labelled enzyme molecules were removed by magnetic separation. Then, the supernatant pH was adjusted to 10.0 by addition of 0.30 mL of glycine–NaOH buffer solution, which was used for spectrofluorometric measurement at 420 nm with an excitation wavelength of 312 nm.[23]

The affecting parameters on the fluorescence intensity may be enzyme concentration and HVA concentration. From previous investigations on the HRP–polymer nanoparticles activity,[22] a pH of 7.5 and room temperature were selected for the assay because the stabilized HRP has its maximum activity under these conditions. Response surface methodology was used for the optimization of enzyme and HVA concentration by using Design Expert® 8.0.4 software.

*Real sample analysis*

As a real sample analysis, the amount of $H_2O_2$ residue in PVP and poly(ethylene glycol) (PEG) as model excipients were determined. For this purpose, 0.1 g of PVP and PEG samples were dissolved in 3 mL of DI water under stirring. 0.3 mL of each sample was added to the sensing reaction tube to determine the amount of $H_2O_2$ in the excipient samples. The $H_2O_2$ content of samples were reported as solution concentration in μmol $L^{-1}$ and also in ppm (μg $g^{-1}$) of the solid form of the sample.

*Enzyme recovery and reusability*

The stabilized HRP–polymer molecules labelled with MNPs can be separated from the sensing mixture by applying an external magnetic field. To investigate the recovery and reusability of the labelled HRP–polymer nanoparticles, the used bio-catalyst was recovered by magnetic separation and washed 3 times with DI water to remove the reaction solution. The recovered catalyst was reused in a subsequent sensing reaction under the same experimental conditions as described before.

*Characterization methods*

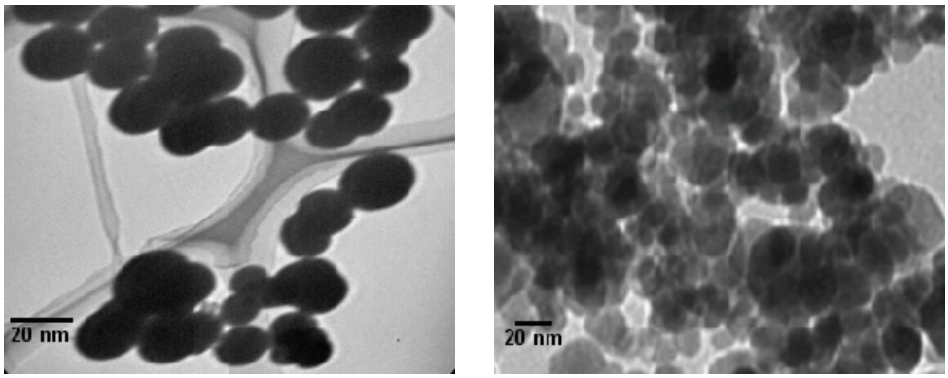
TEM imaging of the samples were determined by means of a CM120 transmission electron microscope, Philips, the Netherlands, using standard carbon-coated grids and 120 kV as the applied voltage. A Cary Eclipse fluorescence spectrophotometer (Varian, Agilent Technologies, Australia) was used for the spectrofluorometric measurements. For protein concentration assay and enzyme activity assessments (with TMB as the substrate[22]), the Lambda 950 UV–Vis spectrometer (PerkinElmer, USA) was used.

## RESULTS AND DISCUSSIONS

### *HRP–polymer nanoparticles preparation*

The HRP molecules were modified by attaching dendron macromolecules to the enzyme surface. Based on previous studies, each fabricated nanoparticle consisted of a single enzyme with five dendron molecules attached to its surface amine groups.[22] Based on TEM images, the average size of the nanoparticles was 14(±2) nm, as shown in Fig. 1a. The life-time and stability analysis revealed that

the dendritic modified HRP showed a significant enhancement in its life-time over 70 days storage at 4 °C (Fig. 2a). The thermal activity of the reengineered enzyme at elevated temperatures up to 80 °C was considerably higher in comparison to that of the free HRP, as shown in Fig. 2b. This result may be due to the attached dendron macromolecules being able to effectively preserve the conformation of the HRP biomolecules against thermal induced unfolding and denaturation caused by movements of the protein moiety around haem.[22] The stability results suggest promising applications of this stabilized enzyme in some interesting fields, such as biosensors.




(a) (b)

Fig. 1. TEM images of a) the fabricated HRP–polymer nanoparticles and b) MNPs attached to the HRP–polymer nanoparticles.

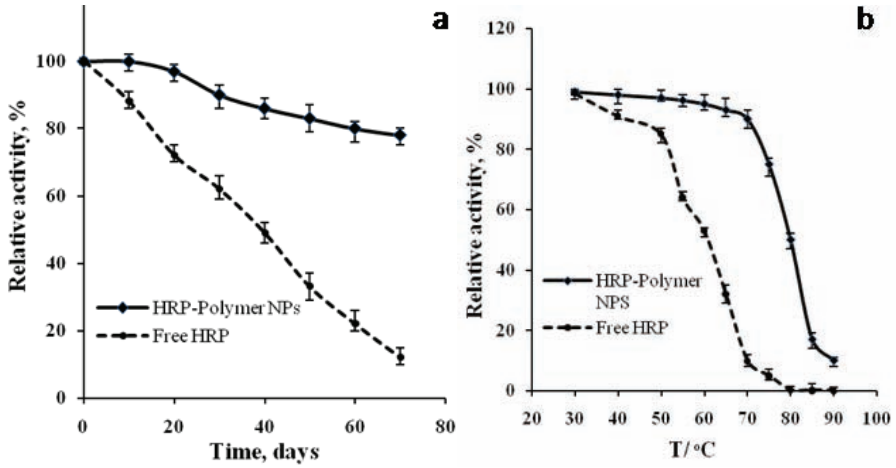


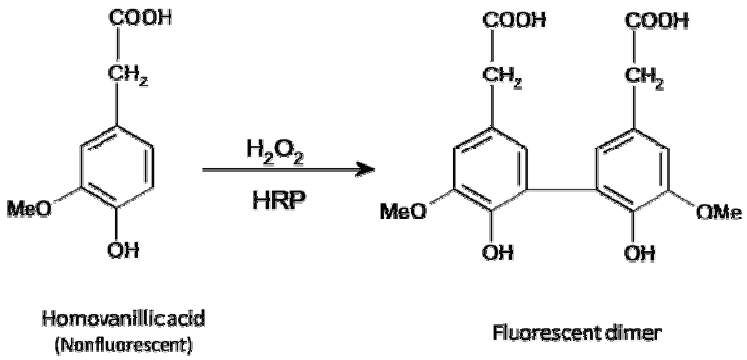

Fig. 2. a) Storage stability and b) thermal stability of the HRP–polymer nanoparticles in contrast to free HRP.

*Magnetic labelling of HRP–polymer nanoparticles*

The $Fe_3O_4$ magnetic nanoparticles (MNPs) with an average size of 8 nm were utilized as magnetic labels for the preparation of a recyclable stabilized biocatalyst. Enzyme activity assays revealed a slight loss of enzymatic activity (less than 4 %) due to the labelling process. The average yield of conjugation for MNPs to dendritic stabilized HRP was 90(±2) %. This labelling yield was obtained from HRP concentration assessment after separation of the enzyme from the reaction mixture and then re-dispersing in 5 mL of PBS. A TEM image of MNPs conjugated to HRP–polymer nanoparticles is shown in Fig. 1b.

*$H_2O_2$ sensing system and its spectral characteristics*

The $H_2O_2$ assay was based on the oxidation of HVA to its fluorescent biphenyl dimer (Scheme 1) in the presence of hydrogen peroxide and peroxidase. As shown in Fig. 3, the product shows the excitation and emission maximum wavelengths at 312 and 420 nm, respectively.[23] The blank samples (HRP–HVA and $H_2O_2$–HVA systems) have no significant fluorescence intensity in comparison with the HRP–HVA–$H_2O_2$ samples. This means that HVA could be oxidized by $H_2O_2$ to a strongly fluorescent dimer in the presence of the HRP enzyme as catalyst. Thus, the determination of $H_2O_2$ could be achieved by using its oxidizing ability, and its concentration is directly proportional to the fluorescence intensity of the HVA dimer. Therefore, the fluorescence intensity of HRP–HVA––$H_2O_2$ system could be used as the basis of the proposed method for detection of $H_2O_2$ traces.




Scheme 1. Oxidation of HVA to a fluorescent dimer by $H_2O_2$ in the presence of HRP.

*Experimental design and optimum conditions*

The concentrations of enzyme (labelled HRP–polymer nanoparticles) and HVA were chosen as independent variables and the fluorescence intensity as a dependent output response variable. The composite system was applied for the response surface methodology and optimization of the parameters.

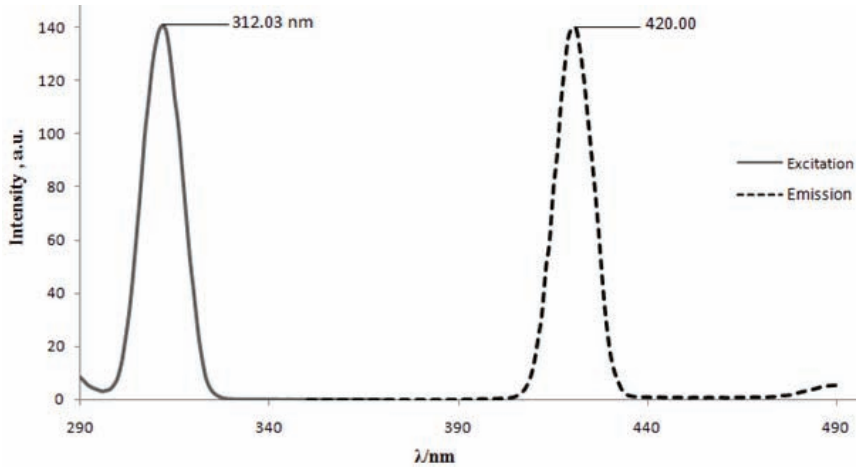



Fig. 3. Excitation and emission spectra of solutions from the systems of HVA/$H_2O_2$/HRP–polymer nanoparticles (labelled). Experimental conditions: $H_2O_2$, $2.0\times10^{-6}$ mol $L^{-1}$; HVA, $100\times10^{-6}$ mol $L^{-1}$; labelled HRP–polymer nanoparticles, 0.4 mg $mL^{-1}$; pH of the reaction solution, 7.5; reaction temperature, 25 °C; reaction time, 5 min; pH of detection solution, 10.0.

The response surface plot resulted from a fitted cubic model (with $R^2$ = = 0.9955) is shown in Fig. 4. This fitted model showed that the fluorescence intensity of this probing system was increased with an increasing enzyme (labelled HRP–polymer nanoparticles) and HVA concentrations. It is revealed that the rate of increase in the intensity was very low for specific levels of HRP and HVA concentrations. This design suggests optimum concentrations of 0.3 mg $mL^{-1}$ and 70 μM for the enzyme and HVA, respectively. These results are based on the minimum enzyme and HVA concentrations consistent with a sufficiently high fluorescence intensity.

*$H_2O_2$ calibration curve*

Under the optimized conditions, this sensing system gave linear responses to $H_2O_2$ in the range $5\times10^{-8}$–$1\times10^{-5}$ mol $L^{-1}$. The calibration curves of $H_2O_2$ detection for different HVA concentrations were obtained by correlating the fluorescence intensity with the $H_2O_2$ concentration. Figure 5 indicates that the fluorescence of the reaction system responded linearly with $H_2O_2$ concentration ($c$, μmol $L^{-1}$) with an HVA concentration of 70 μM and more. The linear regression equation for $H_2O_2$ concentration *vs*. the fluorescence intensity under the optimal conditions was $F = 67.97c + 4.24$ with a correlation coefficient ($R^2$) of 0.997. The detection limit for this probing system was $1.3\times10^{-9}$ mol $L^{-1}$.

*Determination of $H_2O_2$ in real samples*

The application of the prepared sensing system was assessed by the determination of hydrogen peroxide content of 4 excipient samples using the standard

addition method for sample analysis. As shown in Table I, when $H_2O_2$ at three different concentrations was added to the excipient samples, the recoveries of $H_2O_2$ ranged from 96 to 104 % for all samples and the relative standard deviation (*RSD*) was less than 3.5 %. These results showed that this sensing system had sufficient accuracy and reliability and could also be an effective tool for monitoring the hydrogen peroxide in drug excipients and thus useful in the selection of an appropriate one with a smaller amount of $H_2O_2$ impurity.

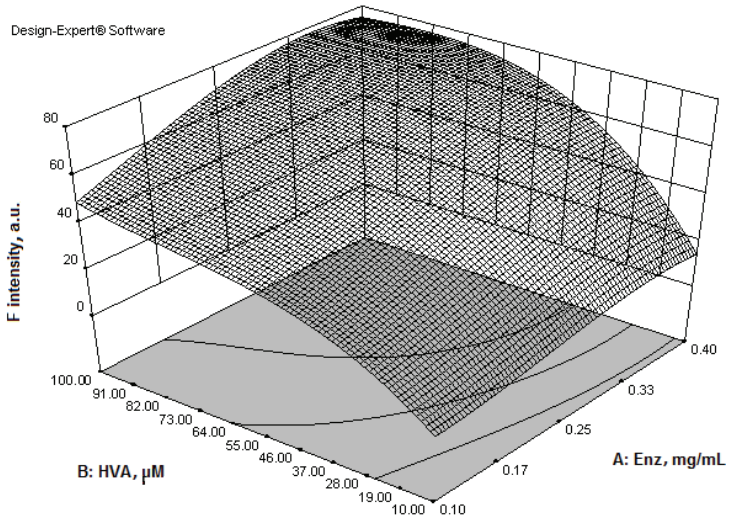



Fig. 4. Surface response plot of the combined effect of concentrations of enzyme and HVA on the fluorescence intensity of the system (enzyme: labelled HRP–polymer nanoparticles).

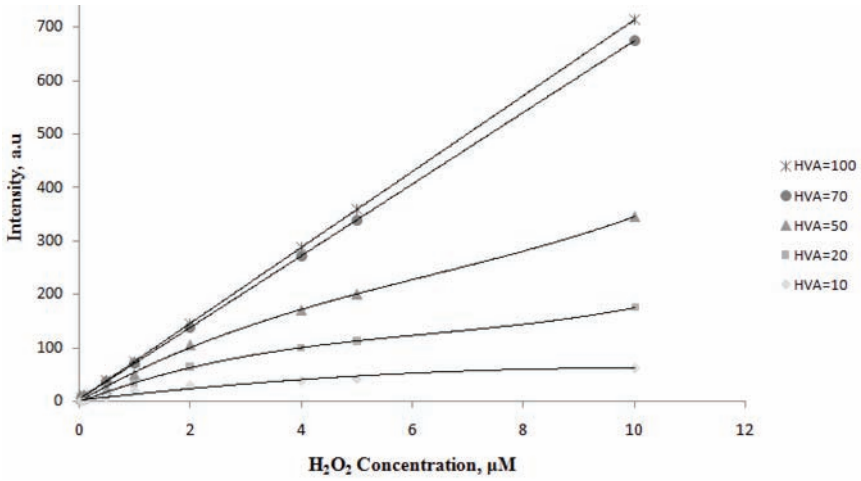


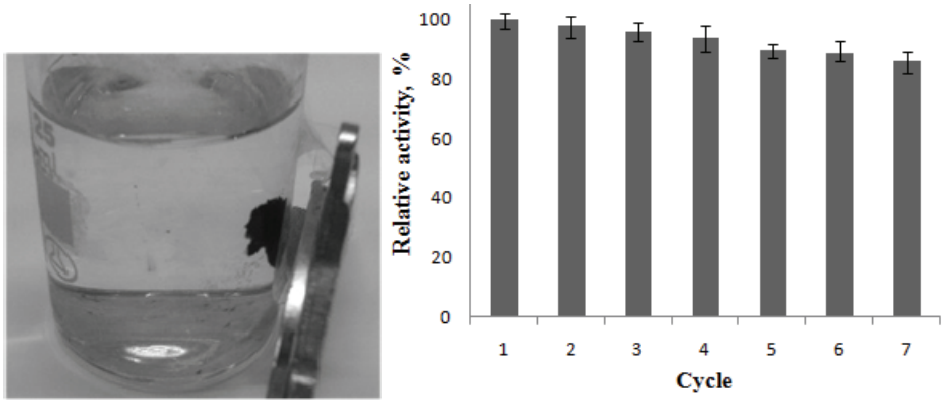

Fig. 5. Calibration curves of HVA-related $H_2O_2$ detection. (labelled HRP–polymer nanoparticles, 0.3 mg $ml^{-1}$ and reaction time, 5min).

Table I. Determination of the $H_2O_2$ content in pharmaceutical excipient samples ($n$ = 5)

| Sample | Added, μmol $L^{-1}$ | Found, μmol $L^{-1}$ | Found, ppm | *RSD*, % | Recovery, % |
|---|---|---|---|---|---|
| PVP-A | 0 | 6.170 | 62.934 | 3.1 | – |
| | 2 | 8.191 | | 2.6 | 101 |
| | 5 | 10.970 | | 3.0 | 96 |
| PVP-B | 0 | 2.642 | 26.948 | 2.4 | – |
| | 2 | 4.683 | | 1.9 | 102 |
| | 5 | 7.795 | | 3.2 | 103 |
| PEG-A | 0 | 0.094 | 0.958 | 1.5 | – |
| | 0.1 | 0.198 | | 1.7 | 104 |
| | 0.5 | 0.588 | | 2.1 | 98 |
| PEG-B | 0 | 0.236 | 2.407 | 2.0 | – |
| | 0.5 | 0.716 | | 3.5 | 96 |
| | 1 | 1.203 | | 2.7 | 98 |

*Enzyme recovery*

The magnetic labelled stabilized enzyme could be easily separated and assessed for its remained catalytic activity (Fig. 6a). To demonstrate the reusability of the stabilized enzyme, the used biocatalyst was recovered by magnetic separation and rinsed thoroughly with distilled water to remove the remaining reaction solution. The recovered enzyme was reused in subsequent sensing reactions under optimal conditions. The relative activity of the reused stabilized HRP for seven first cycles is shown in Fig. 6b, which indicates that the nano reengineered HRP (with dendron macromolecules) exhibited a remarkable stability and could be recovered and reused as the decrease in activity after seven sensing cycles was minimal.




(a) (b)

Fig. 6. a) Magnetic separation of magnetically labelled HRP-polymer nanoparticles and b) relative activity of the reused labelled HRP-polymer nanoparticles.

## CONCLUSIONS

The present study introduced a recyclable biocatalyst consisting of nano-reengineered horseradish peroxidase (by means of dendron macromolecule attachment to the enzyme surface) and attached magnetic nanoparticles, as labelling agents providing recyclability. After stabilization, the HRP showed a remarkable life-time and stability. A simple fluorescence based sensing system for $H_2O_2$ was designed and optimized as a model application for stability and reusability evaluation of fabricated reusable stabilized HRP. The sensing system showed a relatively wide linear response over $H_2O_2$ concentrations from $5\times10^{-8}$ to $1\times10^{-5}$ mol $L^{-1}$ and was successfully applied to monitoring trace levels of $H_2O_2$ residues in PVP and PEG excipients, to ensure their safety and efficacy. The significant stability of HRP–dendron nanoparticles was further displayed in the $H_2O_2$ probing system after 7 recycling times.

## И З В О Д

## МАГНЕТНЕ НАНОЧЕСТИЦЕ СА ИМОБИЛИЗОВАНОМ ПЕРОКСИДАЗОМ ИЗ РЕНА – НАНОБИОКАТАЛИЗАТОР ЗА ВИШЕСТРУКУ УПОТРЕБУ

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У раду је описано испитивање могућности вишеструког коришћења наноносача са имобилизованом пероксидазом из рена у циљу одређивања водоник-пероксида методом флуоресцентне детекције. Дендронски макромолекули су везани за површину ензима техником биоконјуговања. Настале ензим–полимер наночестице, просечне величине 14(±2) nm, имале су значајан полуживот и термичку стабилност. Да би се ензим могао вишеструко користити, ензим–полимер наночестице су обележене магнетним наночестицама, уз принос од 90 %. Овако обележене ензимске честице су испољиле значајну процесну стабилност и могле су се користити до 7 пута у сензорском модел систему. Добијена је линеарна калибрациона права за концентрације водоник-пероксида у опсегу $5\times10^{-8}$ до $1\times10^{-5}$ mol $L^{-1}$, уз детекциони лимит од $1{,}3\times10^{-9}$ mol $L^{-1}$ за сензорски систем под оптималним условима.

(Примљено 30. септембра, ревидирано 26. новембра 2012)

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