Biomacromol. J. Vol. 1, No. 1, 104-112, July 2015.

Biomacromolecular Journal
www.bmmj.org

Simple and Rapid Immobilization of Firefly Luciferase on Functionalized Magnetic Nanoparticles; a Try to Improve Kinetic Properties and Stability

M. Ebrahimi[a,*], S. Hosseinkhani[b], A. Heydari[c] and J. Akbari[c]

[a]*Department of Biochemistry and Biophysics, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran*

[b]*Department of Biochemistry, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran*

[c]*Department of Chemistry, Faculty of Science, Tarbiat Modares University, Tehran, Iran*

(Received 14 December 2014, Accepted 30 April 2015)

ABSTRACT

We expressed and purified a recombinant P. pyralis luciferase with N-terminal His-tags. The silanized Ni or Cu-loaded magnetic particles were prepared and used to assemble the His-tagged P. pyralis luciferase. This enzyme immobilized on functionalized magnetic nanoparticles (MNPs) via electrostatic interactions of His-tag with Ni^{2+}/Cu^{2+} ions on the surface of MNPs using simple one step method. These particles were also used for purification of recombinant luciferase from crude extract of cell lysate. Effect of incubation time and amount of MNPs in bioluminescent activity were investigated to determine optimum condition for immobilization. Several properties of immobilized luciferase were studied and compared with free enzyme. Immobilization has shown different effects on Km for ATP and luciferin. In both immobilized form, Km(ATP) was increased while Km(luciferin) was shown decreases. Optimal temperature of both immobilized luciferase increased to 30 ºC while thermal stabilities have not shown significant differences compared to free enzyme. Both immobilized form inactivated after five consecutive reaction cycles.

Abbreviations: P. pyralis: Photinuspyralis, MNP: Magnetic nanoparticles

Keywords: P. pyralis, Luciferase, Magnetic nanoparticles, Immobilization, Thermostability

INTRODUCTION

Firefly luciferase (EC 1.13.12.7) catalyzes oxidation of luciferin, a benzothiazole compound biosynthesized from cysteine, in the presence of Mg^{2+}-ATP and oxygen [1]. Luciferase used as an important part in several sensitive, simple and convenience assays such as clinical testing [2], drug screening [3], as biosensors for environmental pollutants [4], biomass monitoring and assay of enzymes involving in ATP generation or degradation [5,6]. Sensitivity and precision of bioluminometric assays have been limited due to rapid inactivation of luciferase at moderate temperatures (25-30 ºC) [7,8]. Compared to soluble enzymes, immobilized enzymes have several advantages, such as improved stability, reusability and easier handling.

Immobilization of enzymes on solid support is a very interesting and functional technique was used in various areas of biosciences and biotechnology [9]. Enzyme immobilization has several advantages including easier separation of reaction products, reusability of enzyme, increased enzyme stability due to stabilization of tertiary structure of enzyme and so increased operational lifetime [10,11]. Amongst various methods of immobilization, non-covalent techniques provide the simplest and most rapid method. Furthermore, covalent immobilization of enzymes may be lead to inactivation of the enzyme function. A simple and rapid immobilization method is valuable to generate a more stable and active luciferase for the routine use as a light-emitting sensor in bioassays [12].

In the last decade, magnetic nanoparticles (MNPs) widely reported as an support for immobilization of various bioactive compounds such as proteins, DNA, various

*Corresponding author. E-mail: mehd_ebrahimi@yahoo.com

chemicals, viruses and so on [13,14]. Magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) nanoparticles are becoming as the most desirable and appropriate enzyme carriers, in particular, due to their chemical inertness, size compatibility with enzymes, easy enzyme recovery from the medium under the magnetic force [15] and strong magnetization response [16-18]. Moreover, Silica-coated magnetic nanoparticles can be functionalized in their surface *via* silanol or Si-OH groups [14].

In this work, functionalized Fe_2O_3 magnetic nanoparticles were used to simple and rapid immobilization of recombinant His-Tagged *Photinus pyralis* firefly luciferase. We expressed and purified His-Tagged luciferase and then immobilized on Ni^{2+}/Cu^{2+} containing MNPs. After optimization of MNPs amount and time for immobilization, kinetic, optimal temperature, thermal stabilities and reusability of immobilized luciferase were determined and compared with free enzyme.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Merck (Darmstadt, Germany). ATP, $MgSO_4$ and Tris/HCl were purchased from Sigma Chemical Co (Poole, Dorset, England). D-luciferin potassium salt was purchased from Resem BV (The Netherlands). All the other reagents were of analytical grade and all the solutions prepared with double distilled water.

Preparation of Functionalized Magnetic Nanoparticles

The functionalized magnetic nanoparticles were prepared according to the procedure reported earlier with some modifications [19]. The functionalization procedure was presented schematically in Fig. 1. According to this procedure, magnetite (γ-Fe_2O_3) nanoparticle was synthesized by a chemical co-precipitation technique of ferric and ferrous ions in alkali solution [20,21]. $FeCl_2.4H_2O$ (1.99 g) and anhydrous $FeCl_3$ (3.25 g) were dissolved in deionized water (20 ml) separately, followed by two iron salt solutions being mixed under vigorous stirring (800 rpm). A NH_4OH solution (0.6 M, 200 ml) was then added to the stirring mixture at room temperature and immediately followed by the addition of a concentrated NH_4OH solution (25 w/w%, 30 ml) to maintain the reaction pH between 11 and 12. The resulting black dispersion was continuously stirred for 1 h at room temperature and then heated to reflux for 1 h to yield a brown dispersion. The magnetic nanoparticles were then purified by 3 repeated cycles of centrifugation (3000-6000 rpm, 20 min),

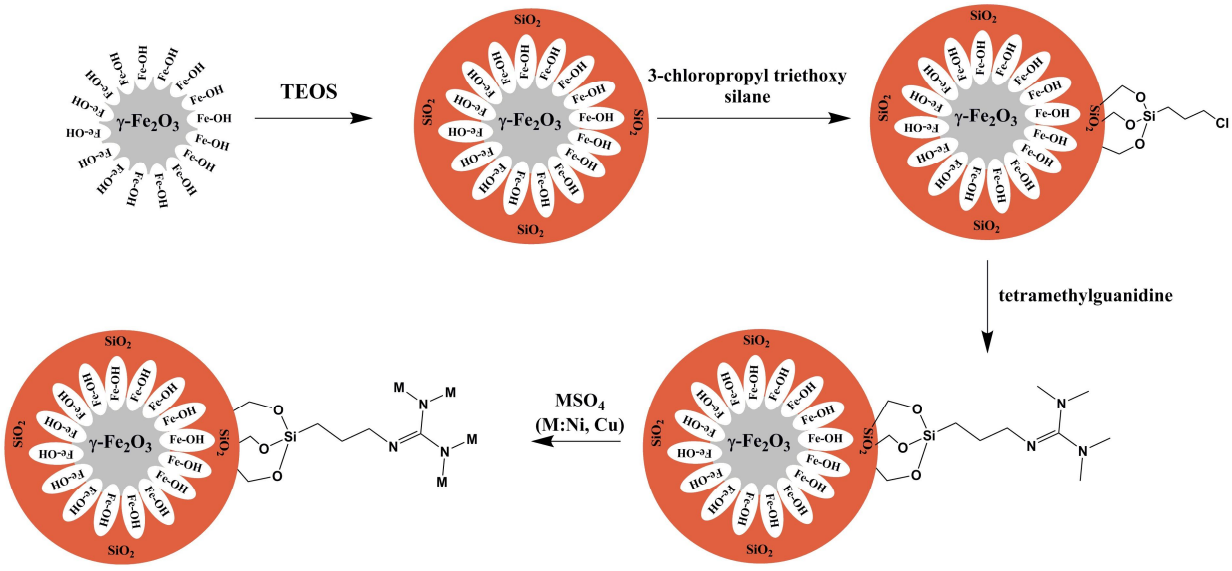

Fig. 1. Schematic representation for surface modification procedure and structure of functionalized Υ-Fe_2O_3 MNPs.

decantation, and re-dispersion, until a stable brown magnetic dispersion (pH 9.4) was obtained.

Surface Modification of the Particles

Coating of a layer of silica on the surface of γ-Fe_2O_3 nanoparticles was achieved by premixing (ultrasonic) a homogenizedpurified nanoparticles (8.5 w/w%, 20 ml) with methanol (80 ml) for 1 h at 40 °C. Concentrated ammonia solution was added, and the resulting mixture was stirred at 40 °C for 30 min. Subsequently, tetraethyl orthosilicate (TEOS, 1.0 ml) was charged to the reaction vessel, and the mixture was continuously stirred at 40 °C for 24 h. The silica-coated nanoparticles were collected by a permanent magnet, followed by washing three times with EtOH, diethyl ether, respectively and dried at 100 °C in vacuum for 24 h. These obtained magnetic nanoparticles and 3-chloropropyl-triethoxy-silane (50 mM) were stirred at 95 °C for 24 h. The product was washed with ether and dried under vacuum. In the next step, the product was stirred with tetramethylguanidine to obtain γ-Fe_2O_3-SiO_2-TMG. A copper sulfate/Nickel sulfate solution (0.1 M) was applied to the silanized particles for 3 min, and then precipitated with an external magnetic field. The functionalized particles were washed five times with excess DI water to remove unbound metal ions. Scanning electron microscopy was used to determine morphological properties of MNPs (Fig. 2).

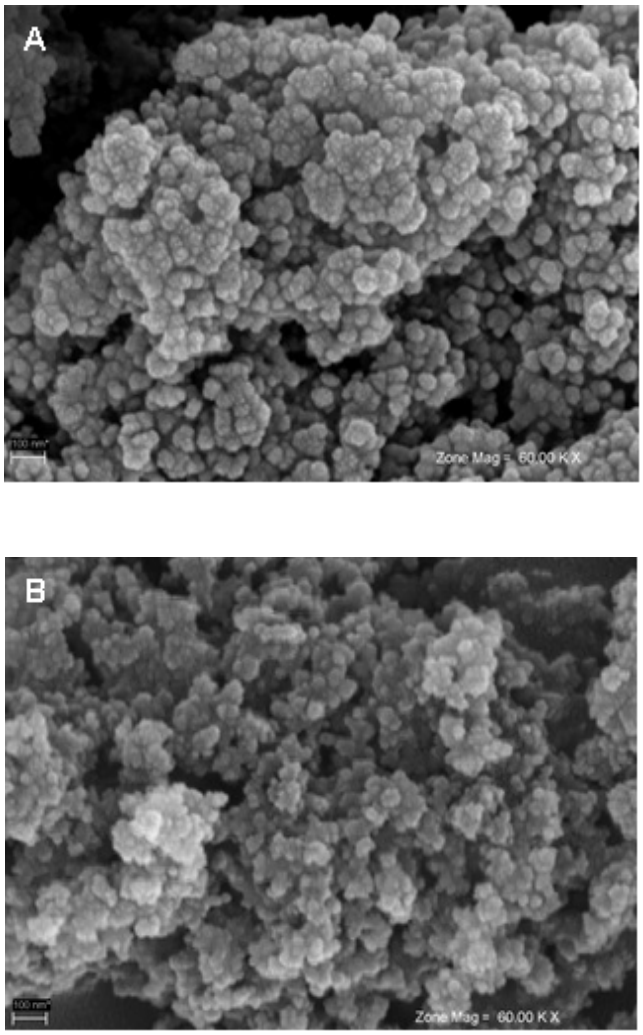

Fig. 2. SEM images of Ni^{2+} (A) and Cu^{2+} (B) containing MNPs. MNPs were agglomerated and made bigger clusters even after dispersed in Tris buffer (pH 7.8).

EXPRESSION, PURIFICATION AND ATTACHMENT OF LUCIFERASE ONTO MNPS

Enzyme Production and Purification

Production of enzyme and purification steps carried out according to previously reported manner [22]. In brief, the *P. pyralis* luciferase was produced and over expressed in *E. coli* cells, strain BL21, using pET expression system. Ni-NTA Sepharose (Qiagen, Inc.) column was used for enzyme purification. The purity of the luciferase was determined by SDS-PAGE and protein concentration was measured by Bradford method [23].

Luciferase Adsorption to Functionalized MNPs

Ionic interaction between Ni/Cu ions and His-Tag was used to adsorption of recombinant luciferase on functionalized MNPs [19]. For this purpose, functionalized magnetic nanoparticles was mixed with the luciferase solution (0.133 µg/µl) at 4 °C and gently shaken for 5-30 min (with 5 min intervals) at the same temperature. An external magnetic field was used to separate MNPs. The pellet was washed with 50 mM Tris-HCl buffer (pH 7.8) three times to remove unbound protein. Bioluminescence was assayed by mixing 25 µl of immobilized enzyme in assay buffer (50 mM Tris-HCl, pH 7.8) with 25 µl of

substrate solution (2 mM luciferin, 10 mM $MgSO_4$, 4 mM ATP and 50 mM Tris-HCl buffer, pH 7.8). Luminescence intensity was recorded with a Sirius single tube Luminometer (Berthold Detection Systems, GmbH) by integration of total light emitted in 10 s. All experiments were repeated three times and mean of repetitions were reported.

To investigate enzyme immobilization, we used MNPs for purification of recombinant luciferase from cell lysate. All the procedure was done similar to purified enzyme adsorption with two exceptions. First, we used cell lysate instead of purified enzyme and in second, desorption of enzyme from MNPs surface was performed using an Immidazol containing buffer (Tris-HCl 50 mM, NaCl 300 mM, Imidazole 250 mM pH 7.8). The supernatant was denoted as "eluent" in further discussion. SDS-PAGE shows SDS-PAGE was performed to determine purity of active fractions and comparison with Ni-NTA Sepharose method (Fig. 3).

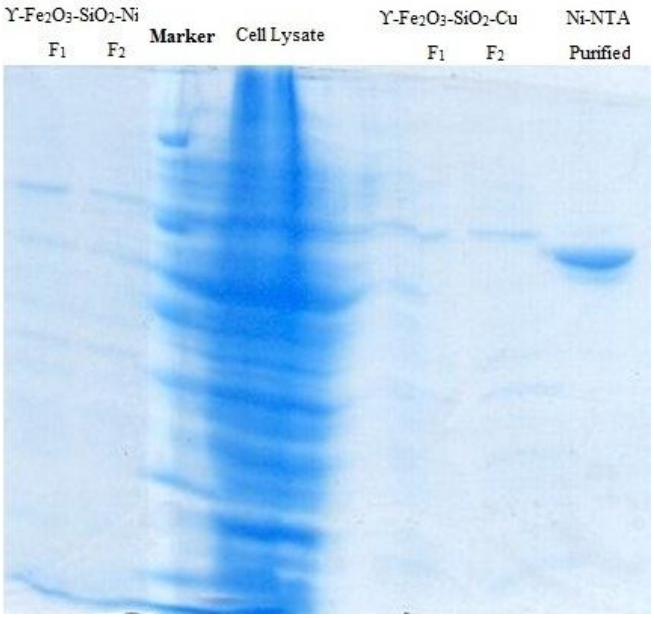

Fig. 3. SDS-PAGE of the particles and Ni-NTA Sepharose elution samples.

Determination of Kinetic Parameters

Kinetic parameters of both immobilized and free luciferase were determined according to previously reported methods [5,7,22]. To estimate luciferin Km, 25 µl of purified enzyme with appropriate concentration (diluted in 50 mM Tris-HCl, pH 7.8) mixed with 25 µl of substrate solution (10 mM $MgSO_4$, 4 mM ATP and 50 mM Tris-HCl buffer, pH 7.8 with various concentrations of luciferin from 0.01-2 mM). The estimation of ATP kinetic constant was performed in a similar way but various concentrations of ATP from 0.03-4 mM were used in substrate solution. The same protocol was used to measuring the Km for immobilized enzyme. Apparent kinetic parameters were calculated by Line weaver-Burk plots.

Optimum Temperature

The optimum temperature of enzyme was gained for both free and immobilized luciferase by measuring the activity at 10-40 ºC by 5 ºC intervals. For this purpose, assay buffer was mixed with substrate solution and incubated in each temperature for 5 min. After this time, the enzymatic reaction was started by adding 25 µl of immobilized and free enzyme. Constant stock solution of immobilized and free enzyme were used during the procedure.

Thermal Stability

Thermal stability of free and immobilized luciferase were measured by incubation of enzyme in assay buffer at 25, 30, 35 and 40 ºC for 5, 10, 15 and 20 min. Before activity measurements, samples were placed on ice for 5 min and remaining activity was determined by addition of substrate solution [1,24]. Rates of inactivation were calculated by a least-squared fit of plots of the log of the remaining activity against time.

RESULTS AND DISSCUTION

Adsorption of Luciferase on MNPs

To gauge the specificity of interactions between enzyme and funtionalized MNPs, the particles were mixed directly with cell lysate containing over expresed recombinant luciferase. After incubation time for binding of enzyme to the particles, an external magnetic field was used to separate particles from crude extract. The particles were washed for three times with washing buffer containing Imidazol.

Eluents were collected in fractions and active fractions analyzed using SDS-PAGE (Fig. 3). Purity of MNPs fractions compared to Ni-NTA Sepharose in Fig. 3 demonstrate high specific interaction between luciferase and particles.

The Effect of MNP on Luminescene Activity

Figure 2 shows that both type of functionalized MNPs were aggregated and made small granular clusters even after dispersed in Tris buffer (pH 7.8). This property can affect efficiency of immobilization. Moreover, electrostatically driven assemblies are often multilayer and can lead to improper alignment of the enzymes on the surface and so their conformational stability is often altered upon binding [19,25-27]. Change in conformation of enzyme will cuase changes in its activity and stability. To prevent this effect two important factors in immobilization (incubation time and amount of MNPs) must be determined as a function of best bioluminescent activity not the amount of immobilized luciferase. To determine optimum time for immobilization, the same concentration of purified enzyme (0.133 µg/µl) was used to immobilization on 1 mg of each MNPs in different time intervals. The bioluminescence activity (RLU/s) as a function of incubation time is shown in Fig. 4. Maximal bioluminescnt activity was reached after 15 and 20 min incubation with Cu and Ni-MNP, respectively. The binding time is shorter than other methods such as biotin carboxyl carrier protein method (30 min) [28]. The bioluminescent activity of Cu-MNP immoblized luciferase was less than Ni-MNP counterparts in all incubation times.

To compare the immobilization capacity of MNPs, the effect of different amounts of both MNPs on the bioluminescent activity were examined. For this porpose, a similar concentration of luciferase (0.133 µg/µl) with different amounts of MNPs were used for immobilization. According to Fig. 5, maximal bioluminescent activities were in 0.25 and 2 mg of Cu and Ni-MNP, respectively. Higher amount of both MNPs were decreased the bioluminescent intensity which my be due to the quenching of light with the unbound MNPs.

Kinetic Properties of Free and Immobilized Luciferase

Depending on the pH, used buffer and assay conditions,

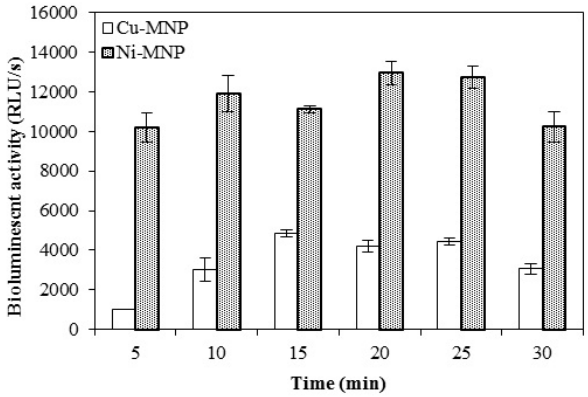

Fig. 4. Effects of incubation time of immobilization in bioluminescent activity. Purified luciferase incubated for different times in 4 ºC and bioluminescent activity measured by mixing the immobilized luciferase with substrate solution in 25 ºC.

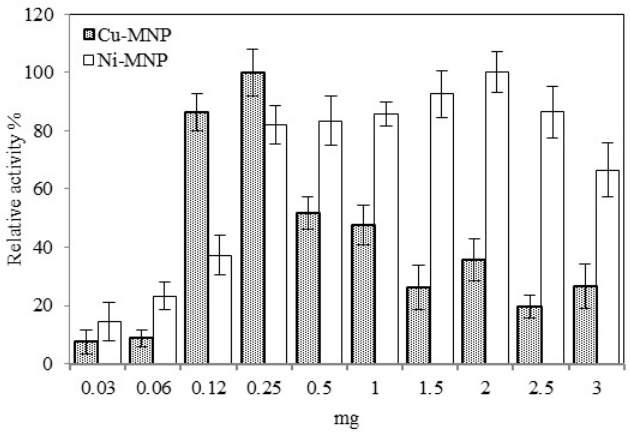

Fig. 5. Effects of mg of MNPs on bioluminescent activity of immobilized luciferase. Activities were compared to maximum activity to determine relative activity.

the Km for ATP and Km for luciferin *Photinus pyralis* firefly lyciferase are in the ranges of 2-125 and 2.4-125 µM, respectively [29,30]. The Km values for free and immobilized luciferase calculated using Line weaver-Burk plot. According to our assay condition, we have examined the Km(ATP) and Km(luciferin) for free enzyme 111 and 10 µM. Km(ATP) for immobilized luciferase for both Cu

and Ni-MNPs were increased to 300 and 200 µM, respectively. In spite of Km(ATP), Km(luciferin) were respectively decreased to 7.5 and 6 for Cu and Ni-MNP. The structural changes induced upon immobilization of enzymes on solid supports often yields some modifications of the kinetics compared to the free enzyme and the affected kinetic behavior is generally diffusion resistance [31]. Studies of firefly luciferase immobilization on different supports demonstrated that Km is dependent to the kind of support used for immobilization. Immobilization of firefly luciferase on epoxy methacrylate polymer and nylon supports leads to decrease in Km(ATP) and increase in Km(luciferin) [32,33] while according to Eu's report [28], the immobilized BCCP-FL has a decrease in Km(luciferin) and an increase in Km(ATP). The influence of mass transfer on the kinetics of immobilized luciferase cannot be ignored. Increasing of Km(ATP) described the failure in mass transfer and dispersion.

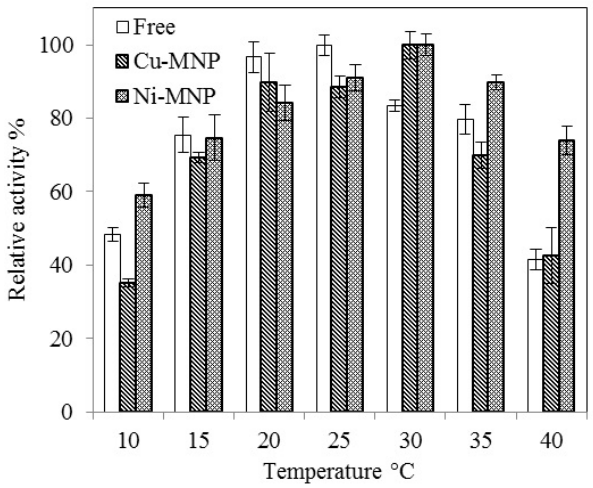

Fig. 6. Effect of temperature on activity of free and immobilized luciferase. The relative activity (%) refers to the percentage of the initial reaction rate obtained by the enzyme in the presence of the above ionic liquids as compared to maximum activity.

Optimum Temperature and Thermal Stability

Low thermal stability of native *Photinus pyralis* firefly luciferase is one of the most important limmiting factors for its use in biosensors. Optimum temperature for native enzyme determined in the range 22-28 ºC and enzyme inactivation occurs in elevated temperatures [7,8]. One of the best known effects of immobilization of enzymes is better thermal stability compared to the free enzyme. Maximal bioluminescent activity of free and immobilized luciferase between different temperatures (in the range of 10-40 ºC) was used to determine the optimum temperature. The optimum teperature of free *Photinus pyralis* firefly luciferase accurately determined at 25 ºC (Fig. 6) [7]. Immobilized luciferase on both Cu and Ni-MNP was shown a 5 ºC increase in optimum temperature. A significantdifference between MNPs was observed at temperatures above 30 ºC; immobilized luciferase on Ni-MNP was less affected by elevated temperatures.

One of the properties that have been generally considered to be improved *via* immobilization is enzyme stability but there are some exceptions [34]. In this study, the thermal stability of immobilized and free luciferase was examined at four different temperatures; 20, 25, 30 and 35 °C (Fig. 7) in 5, 10, 15 and 20 min incubation. In spite of improvements in optimum temperatures of immobilized luciferase, thermal stability slightly improved in comparison with free enzyme. Luciferase immobilized on Ni-MNP retained 18% of its initial activity after 5 min at 20 °C while Cu-MNP immobilized and free luciferase retained nearly 11 and 7% of their original activities, respectively. A significant difference between free and immobilized luciferase was observed with increase in incubation time. Rates of inactivation in free luciferase were more than both immobilized enzyme at 5-20 min. The inactivation pattern was different at 25 °C when free luciferase has more remaining activity in 5 min than immobilized forms. This could be explained by the optimum temperature of free luciferase at this temperature. Upon longer incubation times, rates of inactivation were similar to 25 ºC. An improvement in thermal stability at 35 ºC was observed for Ni-MNP immobilized enzyme. The activity of Cu and Ni-MNP immobilized enzyme have been decreased to nearly 19 and 12% while the free enzyme activity was 7%. Both immobilized and free luciferase completely inactivated at 35 ºC. Kuan [35] reported a similar immobilization method for D-amino acid oxidase (DAO) on magnetic beads. According to this report, thermal stability of immobilized DAO improved significantly (56% remaining activity at

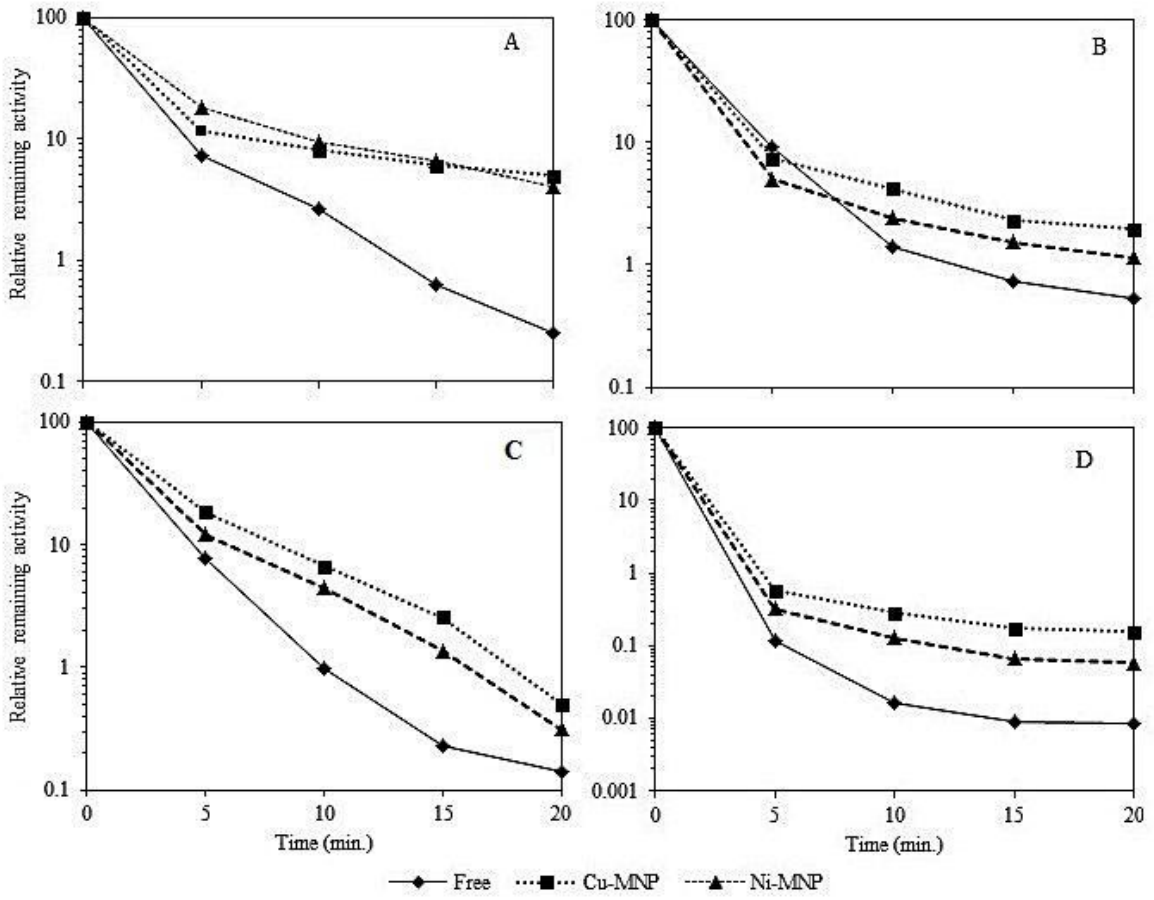

Fig. 7. Thermal stability of free and immobilized luciferase. The stability was determined by incubating at temperatures 20 (A), 25 (B), 30 (C) and 35 °C (D) for 5-20 min followed by 5 min incubation on ice and assaying the activity at 25 ºC.

50 ºC for 1 h). Stabilization of subunits explained as the most important reason for this improvement in thermal stability. In case of firefly luciferase, unfolding of enzyme structure upon heat treatment is the main mechanism of thermal inactivation. Thermal stability of immobilized bacterial luciferase on alkyl-substituted Sepharose 4B was increased with higher number of hydrogen bonds [12]. In the study by Eu, no significant difference in thermal stability was reported between free and immobilized BCCP-FL [28]. As a result, immobilization of luciferase from poly His-Tag cannot stabilize the overall structure of enzyme and therefore thermal stability was not improved.

The reusability of immobilized luciferase on both Cu and Ni-MNP is shown in Fig. 8. Five reactions were performed in succession. A continuous loss of activity was observed; less than 1% of initial activity remained after 5 cycles in both immobilized form. Similar result reported for immobilized BCCP-FL; even in the presence of CoA lost almost all the activity after 4 cycles. The dehydroluciferyl-AMP and oxyluciferin are two important products that strongly inhibit the luciferase [36]. Remaining of these products on the active site may be the most important reason for activity loss in each cycle [28]. The other reason could be the leakage of immobilized luciferase from MNPs. According to a report by *Sonia et al.* a continuous protein leakage observed when *Rg*DAO bound to nickel-chelate matrix [37].

In conclusion, results presented in this communication

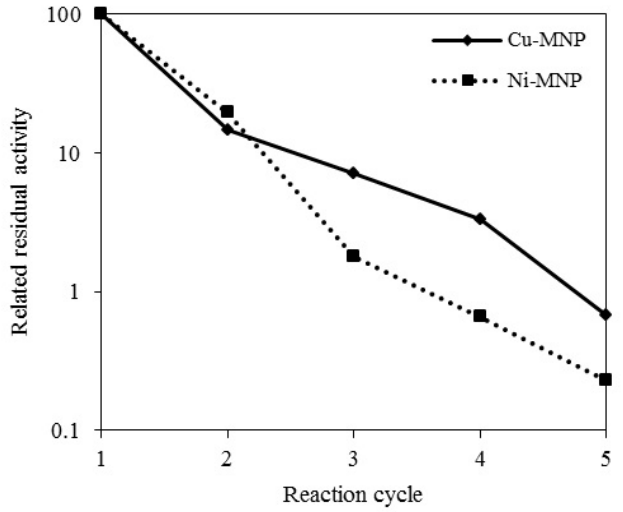

Fig. 8. Reusability of immobilized luciferase. The activities were normalized to the initial value. After each activity measurement, immobilized luciferase was collected with an external magnetic field and after washing (three times) with assay buffer (50 mM Tris-HCl, pH 7.8).

indicate that recombinant His-Tagged *Photinus pyralis* firefly luciferases can readily be immobilized onto functionalized Cu and Ni-MNPs *via* electrostatic interactions. The MNPs were easily separated from reaction mixture using a magnetic field. The incubation time and the amount of MNP are two important factors to give maximum bioluminescent activity of immobilized luciferase. The immobilization of luciferase on MNPs enhanced its optimum temperature but thermal stability slightly increased.

REFERENCES

[1] T. Nakatsu, S. Ichiyama, J. Hiratake, A. Saldanha, N. Kobashi, K. Sakata, *et al*., Nature. 440 (2006) 372.

[2] B.A. Hanna, Methods Enzymol. 133 (1986) 22.

[3] P. Connolly, S.F. Bloomfield, S.P. Denyer, J. Appl. Bacteriol. 75 (1993) 456.

[4] S. Tauriainen, M. Virta, W. Chang, M. Karp, Anal. Biochem. 272 (1999) 191.

[5] F. Ataei, S. Hosseinkhani, K. Khajeh, J. Biotechnol. 144 (2009) 83.

[6] A.K. Campbell, Chemiluminescence: Principles and Applications in Biology and Medicine (Ellis Horwood Series in Biomedicine), VCH/Ellis Horwood, 1988.

[7] M. Mehrabi, S. Hosseinkhani, S. Ghobadi (2008).

[8] S. Ford, F. Leach, Improvements in the Application of Firefly Luciferase Assays, in: R. LaRossa (Ed.), Biolumin. Methods Protoc. SE - 1, Humana Press, 1998: pp. 3-20.

[9] I. Safarik, M. Safarikova, Chem. Pap. 63 (2009) 497.

[10] E. Karakuş, Ş. Pekyardımcı, J. Mol. Catal. B Enzym. 56 (2009) 13.

[11] F. Vaillant, A. Millan, P. Millan, M. Dornier, M. Decloux, M. Reynes, Process Biochem. 35 (2000) 989.

[12] S. Hosseinkhani, R. Szittner, M. Nemat-gorgani, E.A. Meighen, Enzyme Microb. Technol. 32 (2003) 186.

[13] C.C. Berry, A.S.G. Curtis, J. Phys. D. Appl. Phys. 36 (2003) R198.

[14] F. Šulek, M. Drofenik, M. Habulin, Ž. Knez, J. Magn. Magn. Mater. 322 (2010) 179.

[15] D. Lee, K.M. Ponvel, M. Kim, S. Hwang, I. Ahn, C. Lee, J. Mol. Catal. B Enzym. 57 (2009) 62.

[16] Y. Jiang, C. Guo, H. Xia, I. Mahmood, C. Liu, H. Liu, J. Mol. Catal. B Enzym. 58 (2009) 103.

[17] J.H. Jang, H.B. Lim, Microchem. J. 94 (2010) 148.

[18] S.-H. Huang, M.-H. Liao, D.-H. Chen, Biotechnol. Prog. 19 (2003) 1095.

[19] C. Hung, T.R.P. Holoman, P. Kofinas, W.E. Bentley, Biochem. Eng. J. 38 (2008) 164.

[20] H. Honda, A. Kawabe, M. Shinkai, T. Kobayashi, J. Ferment. Bioeng. 86 (1998) 191.

[21] M. Ma, Y. Zhang, W. Yu, H. Shen, H. Zhang, N. Gu, Colloids Surfaces A Physicochem. Eng. Asp. 212 (2003) 219.

[22] M. Ebrahimi, S. Hosseinkhani, A. Heydari, R.A. Khavari-Nejad, J. Akbari, Photochem. Photobiol. Sci. (2012).

[23] M. Bradford, Anal. Biochem. 72 (1976) 248.

[24] N.K. Tafreshi, M. Sadeghizadeh, R. Emamzadeh, B. Ranjbar, H. Naderi-Manesh, S. Hosseinkhani, Biochem. J. 412 (2008) 27.

[25] Z.G. Peng, K. Hidajat, M.S. Uddin, Colloids and Surfaces. 35 (2004) 169.
[26] D.M. Hylton, S.W. Shalaby, R.A. Latour, J. Biomed. Mater. Res. Part A. 73A (2005) 349.
[27] Z.G. Peng, K. Hidajat, M.S. Uddin, Colloids Surfaces B Biointerfaces 33 (2004) 15.
[28] J. Eu, J. Andrade, Luminescence (2001) 57.
[29] J.L. Denburg, R.T. Lee, W.D. McElroy, Arch. Biochem. Biophys. 134 (1969) 381.
[30] M. Yousefi-nejad, S. Hosseinkhani, K. Khajeh, B. Ranjbar, Advances 40 (2007) 740.
[31] S. Hosseinkhani, M. Nemat-gorgani, Enzyme Microb. Technol. 33 (2003) 179.
[32] G. Carrea, R. Bovara, S. Girotti, E. Ferri, S. Ghini, A. Roda, J. Biolumin. Chemilumin. 3 (1989) 7.
[33] G. Carrea, R. Bovara, G. Mazzola, S. Girotti, A. Roda, S. Ghini, Anal. Chem. 58 (1986) 331.
[34] C. Mateo, J.M. Palomo, G. Fernandez-lorente, J.M. Guisan, R. Fernandez-lafuente, Enzyme Microb. Technol. 40 (2007) 1451.
[35] I. Kuan, R. Liao, H. Hsieh, K. Chen, C. Yu, J. Biosci. Bioeng. 105 (2008) 110.
[36] R. Emamzadeh, S. Hosseinkhani, R. Hemati, M. Sadeghizadeh, Enzyme Microb. Technol. 47 (2010) 159.
[37] F. Sonia, P. Loredano, P.S. Mirella, Enzyme Microb. Technol. 29 (2001) 407.