Biosensors and Bioelectronics 25 (2009) 906–912

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Multiplexed immunoassay using the stabilized enzymes in mesoporous silica

Yunxian Piao[a], Dohoon Lee[b], Jinwoo Lee[c], Taeghwan Hyeon[d], Jungbae Kim[e,*], Hak-Sung Kim[a,f,**]

[a] Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Republic of Korea
[b] Korea Institute of Industrial Technology, Cheonan 331-825, Republic of Korea
[c] Department of Chemical Engineering/School of Environmental Science and Engineering, Pohang University of Science and Technology, Pohang, Gyungbuk 790-784, Republic of Korea
[d] National Creative Research Initiative Center for Oxide Nanocrystalline Materials and School of Chemical and Biological Engineering, Seoul National University, Seoul 151-744, Republic of Korea
[e] Department of Chemical & Biological Engineering, Korea University, Seoul 136-701, Republic of Korea
[f] Graduate school of Nanoscience & Technology (WCU), Korea Advanced Institute of Science and Technology, Daejeon 305-701, Republic of Korea

ARTICLE INFO

Article history:
Received 1 July 2009
Received in revised form 28 August 2009
Accepted 1 September 2009
Available online 6 September 2009

Keywords:
Multiplexed immunoassay
NER-LISA (nanoscale enzyme reactor linked immunosorbent assay)
Mesoporous silica
Glucose oxidase
Trypsin

ABSTRACT

Multiplexed immunoassay system was developed using the enzyme-immobilized mesoporous silica in a form of nanoscale enzyme reactors (NERs), which improve the enzyme loading, activity, and stability. Glucose oxidase (GO) and trypsin (TR) were adsorbed into mesoporous silica and further crosslinked for the construction of NERs, and antibody-conjugated NERs were employed for the analysis of target antigens in a sandwich-type magnetic bead-based immunoassay. This approach, called as NER-LISA (NER-linked immunosorbent assay), generated signals out of enzyme reactions that correlated well with the concentration of target antigens. The detection limit of NER-LISA using NER-GO and anti-human IgG was 67 pM human IgG, and the sensitivity was 20 times higher than that of the conventional ELISA using anti-human IgG conjugated GO. Antibody-conjugated NER-GO and NER-TR were successfully employed for the simultaneous detection of two target antigens (human IgG and chicken IgG) in a solution by taking advantage of signals at different wavelengths (absorbances at 570 nm and 410 nm, respectively) from the assays of GO and TR activities, demonstrating the potential of NER-LISA in multiplexed immunoassay. The NER-LISA approach also enabled the successful use of a protease (trypsin), because the NER approach can effectively retain the protease molecules within the mesoporous silica and prevent the digestion of antibodies and enzymes during the whole process of NER-LISA.

1. Introduction

Nanostructured materials offer advantageous features for enzyme immobilization, such as large surface area, controlled nanostructures, conductivity, and magnetic separation. During the last decade, various types of nanobiocatalytic materials have been reported (Kim et al., 2008b), and their potential applications have been demonstrated in various fields such as biosensors (Lee et al., 2005a), biofuel cells, (Kim et al., 2006; Mano et al., 2003; Minteer et al., 2007), magnetoelectrobiocatalysis (Lee et al., 2005c; Willner et al., 2007), trypsin digestion in proteomic analysis (Fan et al., 2005; Kim et al., 2009; Shui et al., 2006; Zuo et al., 2006), and enzyme-linked immunosorbent assay (ELISA) (Ambrosi et al., 2007; Munge et al., 2005; Piao et al., 2009; Tang et al., 2008; Wang et al., 2004; Yu et al., 2006). Especially, efforts have been made to enhance the sensitivity of conventional ELISA by replacing an antibody-conjugated enzyme molecule with nanobiocatalytic materials containing a large number of enzyme molecules (Ambrosi et al., 2007; Munge et al., 2005; Tang et al., 2008; Wang et al., 2004; Yu et al., 2006). However, most of them employed conductive nanomaterials for electrochemical detection with no serious consideration of enzyme stability, which is critical and important for the fidelity of ELISA signals.

Recently, various nanobiocatalytic approaches have been reported to stabilize the enzyme activities in an unprecedented way, opening up new possibilities of practical enzyme applications (Kim et al., 2008b; Lee et al., 2007; Wang et al., 2001). As an example of nanobiocatalytic enzyme stabilization, we have reported the approach of nanoscale enzyme reactors (NERs), in which the enzyme adsorption into mesoporous materials with the bottle-neck pore structure was followed by the chemical crosslinking of adsorbed enzyme molecules. The NER approach could stabilize the enzyme activity and maintain high enzyme loading by preventing the enzyme leaching via a ship-in-a-bottle effect (Kim et al., 2005; Lee et al., 2005b). In addition, NERs showed a good resistance against proteolytic digestion (Kim et al., 2007), which is very useful for the successful applications of NERs because proteases

* Corresponding author. Tel.: +82 2 3290 4850; fax: +82 2 926 6102.
** Corresponding author. Tel.: +82 42 869 2616; fax: +82 42 869 2610.
E-mail addresses: jbkim3@korea.ac.kr (J. Kim), hskim76@kaist.ac.kr (H.-S. Kim).

0956-5663/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2009.09.005

are ubiquitous and hamper the successful applications of enzymes due to their autolytic activities or by digesting the other protein molecules.

In the present work, we demonstrate a multiplexed immunoassay system using the enzyme-immobilized mesoporous silica in a form of nanoscale enzyme reactors (NERs). The use of NERs is expected to not only enhance the detection sensitivity of ELISA due to their high enzyme loading and activity, but also improve the fidelity of ELISA results due to highly stable enzyme activity of NERs. With these advantageous features of NER-linked immunosorbent assay (NER-LISA), the dual immunoassay was attempted using NERs of glucose oxidase (GO) and trypsin (TR). NER-GO and NER-TR were grafted with anti-human IgG (anti-hIgG) and anti-chicken IgG (anti-cIgG), respectively. Then, the sandwich-type assays using antibody-conjugated magnetic beads were performed in a single solution to assess the sensitivity and the stability of NER-LISA system in the simultaneous detection of human IgG (hIgG) and chicken IgG (cIgG). This study, for the first time, demonstrated a successful use of a protease (trypsin as a model protease) in the approach of ELISA, which will give more flexibility in selection of the signaling method based on a large array of substrate-protease combinations.

2. Experimental

2.1. Chemicals and materials

Glucose oxidase (GO), *o*-dianisidine dihydrochloride, D-glucose, peroxidase, trypsin (TR), N_α-benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BAPNA), *N-tert*-butyloxycarbonyl (Boc)–Gln–Ala–Arg-7–amido-4–methylcoumarin (AMC), glutaraldehyde (GA, 25%), goat anti-human IgG (Fab specific, anti-hIgG), goat anti-human IgG (Fc specific, anti-hIgG), rabbit anti-chicken IgG (against whole molecule, anti-cIgG), human IgG (hIgG), chicken IgG (cIgG), ethanolamine hydrochloride, ammonium sulfate, and sodium cyanoborohydride (5.0 M solution in aqueous ~1 M NaOH) were purchased from Sigma–Aldrich (USA). Sodium meta-periodate and desalting column for purification were purchased from Pierce (USA). Dynabead M-270 Epoxy (2.8 μm diameter) was purchased from Invitrogen (Invitrogen Dynal, Norway).

2.2. Preparation of MCF (Mesocellular Foam) silica

MCF silica was prepared following the reported procedures (Schmidt-Winkel et al., 1999), and functionalized with aminopropyl groups by refluxing of (3-aminopropyl) trimethoxysilane in toluene. MCF silica (1 g) was incubated with 2% v/v 3-aminopropyltriethoxysilane (APTES) solution in dry toluene (10 ml) and the mixture was refluxed under an inert argon atmosphere for 15 h. The aminopropyl group anchored material was collected by filtration, washed with acetone, and dried overnight at 80 °C under vacuum. Aminopropyl group-functionalized MCF (amino MCF, 50 mg) was washed three times with distilled water and three times with 0.1 M sodium phosphate buffer (pH 7.0). The final concentration of washed MCF was 9.2 mg mL^{-1} and it was stored at 4 °C.

2.3. Preparation of NERs

Briefly, MCF silica (6 mg) was incubated in the GO solution (4 mg mL^{-1} in 0.1 M sodium phosphate buffer, pH 7.0) under shaking (250 rpm) for 30 min. After the enzyme adsorption, it was incubated in the GA solution (0.5% in 0.1 M sodium phosphate buffer, pH 7.0) for 1 h. The un-reacted aldehyde groups were capped by using Tris buffer (0.1 M, pH 8.0). NER-GO was washed thoroughly by using sodium phosphate buffer, and stored at 4 °C. NER-TR (MCF-TR) was also prepared following a similar procedure.

2.4. Preparation of NER/antibodies

Antibodies were conjugated onto NER-GO or NER-TR via amine–amine coupling by GA. Briefly, the NER-GO, which was not blocked by the Tris buffer, was incubated in the anti-hIgG solution (Fc specific, 1 mg mL^{-1} in phosphate buffer) at 25 °C for 1 h. After a brief washing with Tris buffer (0.1 M, pH 8.0), the NER-GO/anti-hIgG was incubated in the Tris buffer under shaking (250 rpm) for 1 h. Finally, the NER-GO/anti-hIgG was washed three times with PBS (phosphate buffered saline; 10 mM phosphate, 137 mM NaCl, and 2.7 mM KCl, pH 7.4), re-suspended in skimmed milk solution (1% in PBS), and stored at 4 °C. NER-TR/anti-cIgG was prepared by following a similar procedure. The protein concentration was measured by the Bradford method (Bradford, 1976).

2.5. Preparation of anti-hIgG/GO conjugate

Anti-hIgG/GO conjugate was prepared by coupling the antibodies to glycosylated GO that was oxidized to produce aldehyde groups for reductive amination coupling (Hermanson, 1996). Briefly, GO (10 mg) and sodium meta-periodate (5 mg) were dissolved in 0.1 M sodium phosphate buffer (1 mL, pH 7.0) and incubated in the dark for 30 min at room temperature, followed by purification with desalting column. After concentration using a centrifugal filter (Centricon YM 100, Millipore) to 5.74 mg mL^{-1} in 0.2 M sodium carbonate buffer (pH 9.6), the oxidized GO (0.6 mL) was mixed with anti-hIgG (Fc specific, 1.74 mg in 0.3 mL of 0.2 M sodium carbonate buffer, pH 9.6), which resulted in two-time molar excess of GO over IgG. After incubation for 2 h, 5.0 M sodium cyanoborohydride in ~ 1 M NaOH (9 μL) was added, and the solution was further incubated for 30 min. Then, the reaction was stopped by adding 1 M ethanolamine hydrochloride solution (45 μL, pH 9.6). Finally, anti-hIgG/GO conjugate was purified using a desalting column, concentrated with the centrifugal filter, and stored at 4 °C.

2.6. Enzyme activity and stability

The GO assay kit from Molecular Probes (Invitrogen, USA) was used to measure the GO activity. Briefly, the sample solutions containing GO were diluted in 50 mM sodium phosphate buffer (pH 7.4). Standards and sample solutions of GO (50 μL each) were loaded into wells of a 96-well microplate. Then, fresh assay solution (50 μL; 100 μM Amplex Red, 0.2 U mL^{-1} horseradish peroxidase, and 100 mM glucose) was added to each microplate well, containing GO sample, to initiate chromogenic reaction. After incubation at room temperature in the dark for 30 min, the reaction product (resorufin) was quantified by measuring the absorbance at 570 nm. The GO activity was determined from the standard curve, where one unit (U) was defined as the amount of GO that oxidize 1.0 μmole of β-D-glucose to D-gluconolactone and H_2O_2 per min at 35 °C and pH 5.1. Activities of the anti-hIgG/GO conjugate and the NER-GO were also determined by following the same procedure after appropriate dilution. The TR activity was assayed by the hydrolysis of L-BAPNA (0.1 mg mL^{-1}) and Boc–Gln–Ala–Arg–AMC, which could be monitored by measuring the increase in absorbance at 410 nm and emission at 440 nm (excitation at 380 nm), respectively.

The enzyme stability was assessed by measuring the residual enzyme activities at specific time points, and the relative activity was calculated from the ratio of residual activity to initial activity.

2.7. Preparation of antibody-conjugated MB

Magnetic beads (MB) modified with anti-hIgG (Fab specific) were prepared according to the manufacturer's instruction. Briefly, epoxy-coated magnetic dry beads from Invitrogen Dynal (40 μL, 10^9 beads mL^{-1} in 0.1 M sodium phosphate buffer, pH 7.4), after two times washing, were mixed with anti-hIgG (40 μL, 1 mg mL^{-1} in PBS, pH 7.4) and 3 M ammonium sulfate solution (40 μL, in 0.1 M sodium phosphate buffer, pH 7.4). After incubation at room temperature under tilted shaking for 24 h, the MB/anti-hIgG was washed twice with PBS and twice with skimmed milk solution (0.5% in PBS). Finally, the MB/anti-hIgG was re-suspended in fresh skimmed milk solution and stored at 4 °C. MB/anti-cIgG was prepared in the same way using 1 mg mL^{-1} of anti-cIgG.

2.8. NER-LISA or ELISA

For performance test of NER-LISA, the MB/anti-hIgG (10^7 beads mL^{-1}) was washed three times with wash buffer (0.05% Tween 20 and 0.5% skimmed milk in PBS), and incubated in different concentrations of hIgG (50 μL each, in wash buffer) with tilted and mild shaking for 1 h. After three-time washing, it was incubated with 100 μg mL^{-1} of NER-GO/anti-hIgG, corresponding to 1.8 U mL^{-1} of the GO activity, in 50 μL of wash buffer for 2 h. Following three-time washing, the immunocomplex was incubated in 100 μL working solution (50 μM Amplex Red, 0.1 U mL^{-1} HRP, and 50 mM glucose) for 30 min. The absorbance change at 570 nm was measured using a microplate reader (Infinite M200 Megellan, TECAN) after the supernatant from magnetic separation was collected and transferred to the 96-well microplate. The whole immunoassay process was carried out at room temperature and all the shaking steps were performed in Intelli Mixer (MyLab™ SLRM-2 M). A magnetic separator (Magna Rack™, Invitrogen) was used for the bead separation, and it generally took less than one min to completely collect the magnetic beads. NER-LISA with NER-TR was performed by following the same procedure. The TR-catalyzed hydrolysis of L-BAPNA and Boc–Gln–Ala–Arg–AMC were measured by the increase of absorbance at 410 nm and the emission at 440 nm (excitation at 380 nm), respectively.

For a comparative study, the conventional ELISA was performed using the similar procedure to NER-LISA with NER-GO. In the step of secondary antibody addition, the same volume (50 μL in wash buffer) of excess GO/anti-hIgG conjugate (1:10 diluted from stock solution; corresponding to 114.6 U mL^{-1} of the GO activity) was added instead of NER-GO/anti-hIgG. The GO/anti-hIgG conjugate has a specific activity of 254.8 U mg^{-1} of GO, which represents 60% of free enzyme.

2.9. Simultaneous signal generation by NER-GO and NER-TR

A mixture of NER-GO (100 μg mL^{-1}, 16 wt% loading, 33% activity recovery) and NER-TR (1 mg mL^{-1}, 48 wt% loading, 3% activity recovery) was mixed with the substrate solution containing Amplex Red (50 mM), HRP (0.1 U mL^{-1}), glucose (50 mM), and L-BAPNA (0.1 mg mL^{-1}), and shaken (250 rpm) at room temperature. At intervals, aliquots were withdrawn, and filtered using centrifugal filters (0.22 μm), and the absorbance spectra were obtained using a spectrophotometer. The same experiment was performed using NER-GO or NER-TR, individually.

2.10. NER-LISA for simultaneous detection of dual target antigens

The mixture of MB/anti-hIgG (10^7 beads mL^{-1}) and MB/anti-cIgG (10^7 beads mL^{-1}) was incubated with hIgG and cIgG under tilted and mild shaking for 1 h. After three-time washing, the samples were incubated with NER-GO/anti-hIgG (100 μg mL^{-1} in wash buffer) and NER-TR/anti-cIgG (100 μg mL^{-1} in wash buffer) under shaking for 2 h. After thorough washing, the immunocomplex was mixed with freshly prepared dual-substrate solution (100 μL; 50 mM Amplex Red, 0.1 U mL^{-1} HRP, 50 mU glucose, and 0.1 mg mL^{-1} L-BAPNA), and the absorbance at both 570 nm and 410 nm was measured.

3. Result and discussion

3.1. Preparation and characterization of NER-linked antibodies (NER/antibodies)

GO (dimension: 5.2 nm × 6.0 nm × 7.7 nm) (Hecht et al., 1993) was immobilized in the amine-functionalized MCF (Mesocellular Foam) silica (Schmidt-Winkel et al., 1999), which has 25.5 nm mesocellular pores connected with 11.8 nm window mesopores (See Fig. S1 in Supplementary Information). NER of GO (NER-GO) was prepared by crosslinking pre-adsorbed GO molecules via glutaraldehyde (GA) treatment, which forms covalent linkages between the amine groups on the surface of GO molecules. As a result, crosslinked GO aggregates in the main mesocellular pores (25.5 nm) cannot leach out through the smaller window mesopores (11.8 nm) (Fig. 1A). In addition to this ship-in-a-bottle effect, the GA treatment also formed covalent linkages between some of enzyme molecules and the inner surface of amine-functionalized mesopores, which would further prevent the enzyme molecules from leaching out of the MCF silica. NER-GO in MCF showed an impressive stability, maintaining the initial activity with a negligible loss at room temperature under shaking condition (200 rpm) for more than two weeks. The GO loading of NER-GO was 14% (w/w, silica), and the specific activities of free GO and NER-GO were 400 and 133 U mg^{-1} of GO, respectively. The activity recovery of 33% can be explained by several factors such as the activity loss during immobilization process and washing steps, and the mass transfer limitation of NER-GO. The other way around, the present activity recovery also indicates that the mass transfer limitation of NER-GO is not so severe considering the chemical crosslinking of enzyme molecules and fully packed mesopores with enzyme molecules. Right after the GA treatment, anti-hIgG antibody was covalently attached on the surface of mesoporous silica to prepare NER-GO/anti-hIgG, and the final loading of antibody was 8% (w/w, silica).

NER of trypsin (NER-TR) was also prepared following a similar procedure. NER-TR stabilized the TR activity by maintaining its initial activity at room temperature under shaking condition (200 rpm) for more than 10 days, which is not possible with a soluble form of TR due to the TR autolysis (See Fig. S2 in Supplementary Information). NER-TR/anti-cIgG was also prepared in the same way as that of NER-GO/anti-hIgG, and the final loading of antibody was 11% (w/w silica).

3.2. NER–linked immunosorbent assay (NER-LISA) with NER-GO

A magnetic bead-based assay was designed to take advantage of the high activity and stability of NERs for bio-detection (Fig. 1B). Capture antibodies (anti-hIgG and anti-cIgG) were attached to the epoxy-functionalized magnetic beads to facilitate the capture and the separation of the target molecules, hIgG and cIgG, respectively. The detection antibodies (anti-hIgG and anti-cIgG) were attached to the NERs as described above. Then, the target antigens (hIgG and cIgG) were captured by antibodies on magnetic beads, and further incubated with the NER/antibodies for target-specific sandwich binding. After the magnetic separation, the amount of target antigens was quantified by measuring the absorbance increase due to the colorimetric reactions of bound NERs. Fig. 2A shows the time-dependent increase of absorbance at 570 nm during the GO assay

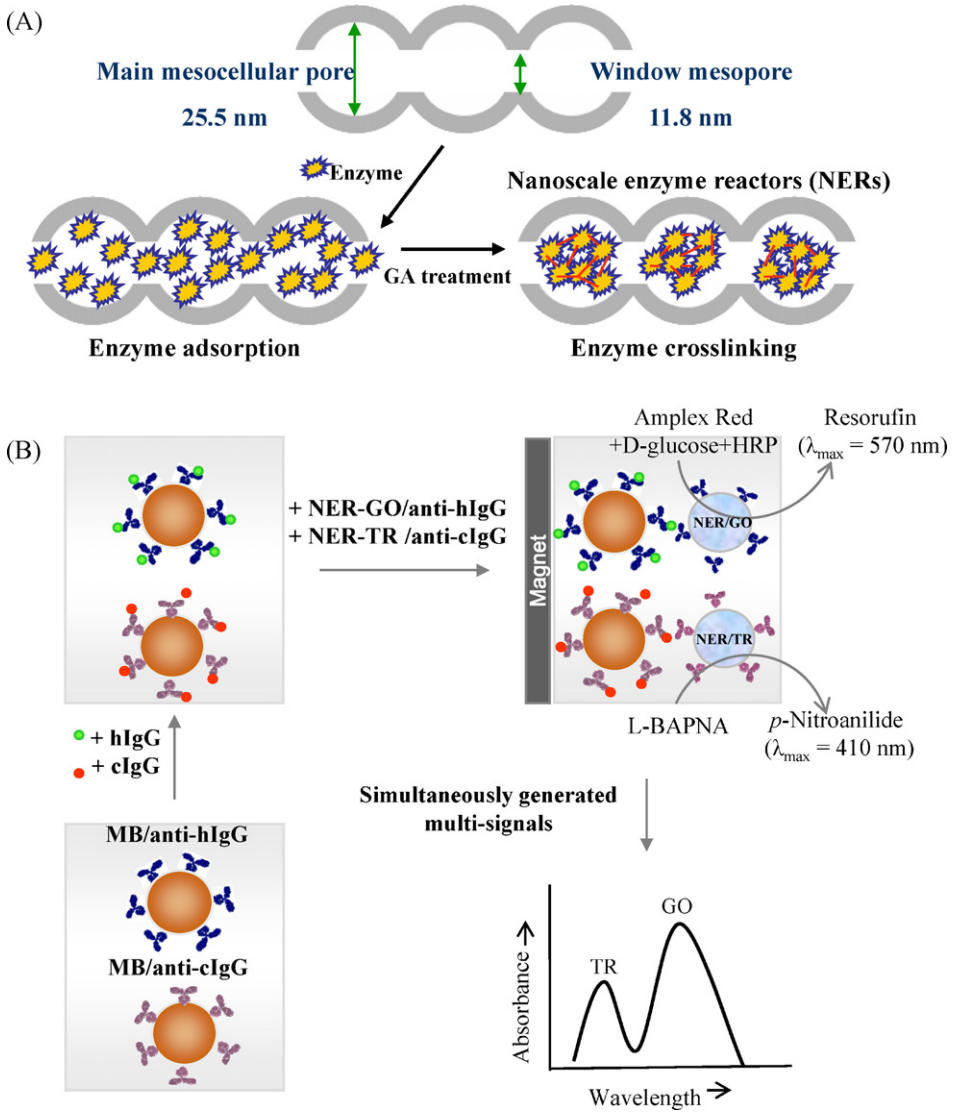

Fig. 1. (A) Diagram depicting the procedure for NER preparation (nanoscale enzyme reactor in MCF silica) (B) Schematic representation of the magnetic bead-based simultaneous dual target immunoassay using NER-GO/anti-hIgG and NER-TR/anti-cIgG (MB: magnetic bead, hIgG: human IgG, cIgG: chicken IgG).

in a NER-LISA detection of 10 μg mL^{-1} hIgG. This result suggests that the NER-LISA shows the same mechanism of signal amplification resulting from the enzymatic reaction as that of conventional ELISA.

The specificity of NER-LISA was tested with two control samples containing no antigen and cIgG, respectively (Fig. 2B). The signals of NER-LISA with these two control samples, represented by the absorbance increase (ΔA_{570}) from the GO assay for 10 min, were negligible and much smaller than the signal resulting from NER-LISA with the target antigen sample (hIgG). This indicates that the NER-LISA using NER-GO/anti-hIgG is specific enough to selectively detect the target antigen (hIgG). The NER-LISA with NER-GO/anti-hIgG showed a linear correlation between the hIgG concentration and the detection signal (ΔA_{570} after 10 min reaction) over the concentration range of 10 ~ 10,000 ng mL^{-1} (Fig. 2C) in a log–log scale. The resulting regression equation was log (A_{570}) = −2.390 + 0.510 log (Concentration of hIgG in ng mL^{-1}) with the correlation coefficient of 0.995. NER-LISA could detect hIgG as low as 10 ng mL^{-1}, which corresponds to the 67 pM of hIgG (~150 kDa). It is anticipated that the sensitivity and the detection limit of the NER-LISA can be further improved by more rigorous control of the NER/antibody preparation and the background signals from non-specific bindings (Oh et al., 2007; Kim et al., 2008a).

For the comparison of NER-LISA with the conventional ELISA, GO was directly attached to an anti-hIgG and used for the detection of hIgG (10 μg mL^{-1}). Under the same assay conditions, the signal of NER-LISA was 20 times higher than that of the conventional ELISA (Fig. 3). This reveals that the NER-LISA approach enables more enzymes to participate in the signal amplification due to the high enzyme loading of NERs.

3.3. NER-LISA with NER-protease

Until now, proteases could not be used in the conventional ELISA approach due to their autolytic and proteolytic activities, leading to the digestion of proteases and conjugated antibodies. However, the NER approach can effectively retain the protease molecules within the mesoporous silica and thus control the proteolysis (See Fig. S2 in Supplementary Information). This feature of the NER approach can open up a new potential of NER-LISA, enabling the use of numerous proteases and their substrates. To demonstrate the use of NER-proteases in the NER-LISA, trypsin (TR) was selected as a model protease, since the TR activity can be measured easily

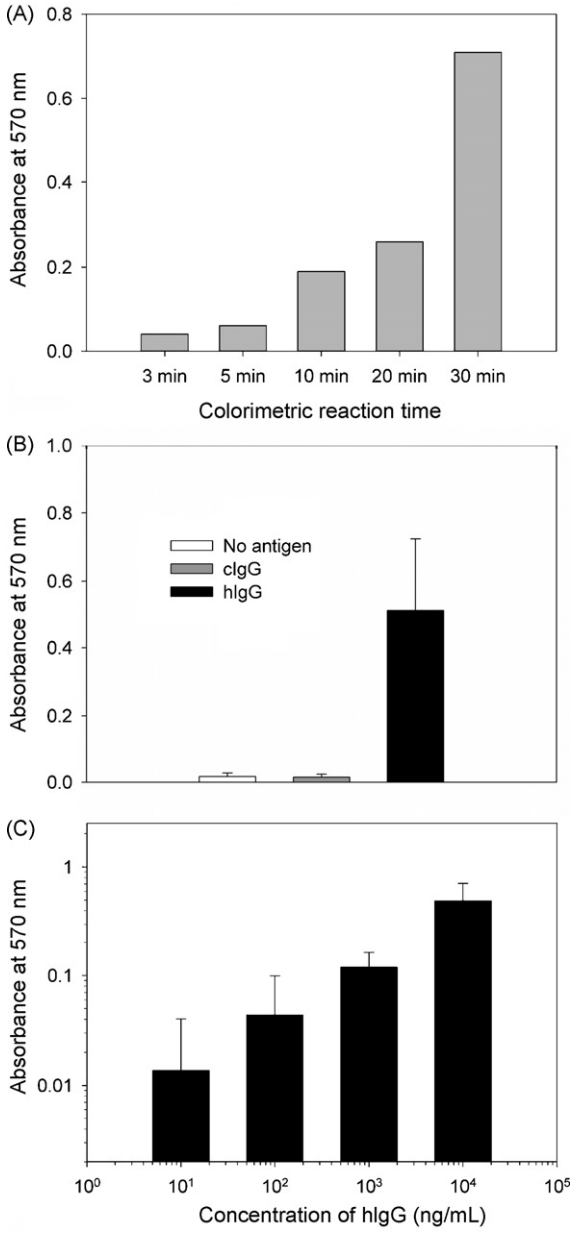

Fig. 2. (A) Time-dependent absorbance increase by NER-GO/anti-hIgG–hIgG–MB/anti-hIgG immuno-complex in the NER-LISA. (hIgG: 10 μg mL^{-1}) (B) Specificity of the NER-LISA in the detection of human IgG (hIgG: 10 μg mL^{-1}). The signals are negligible when no or irrelevant antigen (cIgG: 10 μg mL^{-1}) were used instead. (C) Correlation between the signal of NER-LISA and the target antigen concentration for the detection of hIgG using NER-GO/anti-hIgG and MB/anti-hIgG. The background signal, obtained from no-antigen control reaction, has been subtracted from each value. The error bars indicate standard deviations in triplicate experiments. Incubation time for colorimetric enzyme assay in both (B) and (C) was 10 min.

using a substrate, N_α-benzoyl-L-arginine 4-nitroanilide (L-BAPNA), which increases absorbance at 410 nm upon hydrolysis without interfering with the GO assay based on the ΔA_{570}.

NER-LISA was performed for the detection of cIgG by using NER-TR/anti-cIgG and MB/anti-cIgG. A good linear correlation could be obtained between the cIgG concentration and the detection signal (ΔA_{410} after 30 min of TR-catalyzed L-BAPNA hydrolysis reaction), with the detection limit of 25 ng mL^{-1}, corresponding to the 140 pM of cIgG (~180 kDa) (Fig. 4). The detection signal could also be significantly enhanced by simply increasing the reaction time of colorimetric enzyme assay (See Fig. S3 in Supplementary Information).

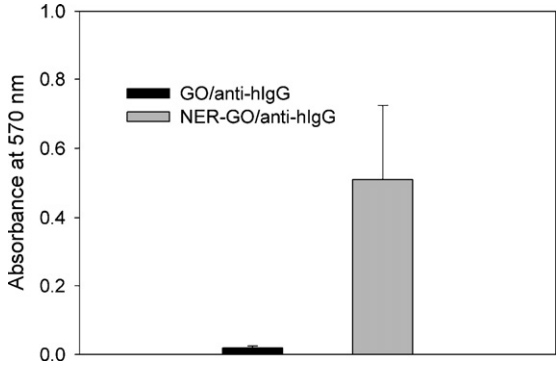

Fig. 3. The comparison of NER-LISA with conventional ELISA in the magnetic bead-based sandwich immunoassay for the detection of hIgG (10 μg mL^{-1}) using NER-GO/anti-hIgG or GO/anti-hIgG conjugate, respectively. The error bars indicate standard deviations in triplicate experiments.

Even though TR is a protease, NER-LISA with the NER-TR demonstrated a similar success to that with NER-GO. This result demonstrates the potential of NER-LISA to complement the ELISA approach with a long list of proteases and their versatile assay methods.

NER-LISA with the NER-TR was also performed by using the fluorescence assay, which is generally known to be more sensitive than the colorimetric assay (See Fig. S4 in Supplementary Information). A fluorogenic substrate, Boc–Gln–Ala–Arg–AMC, was used and, sensitivity of the NER-LISA was improved as anticipated. The detection limit was 2 ng mL^{-1} (11.2 pM cIgG) and the improvement by one order of magnitude was made compared to the colorimetric assay using L-BAPNA (Fig. 4).

3.4. NER-LISA for simultaneous detection of dual target antigens

The simultaneous detection of multiple target antigens using an ELISA approach requires the orthogonal measurement of enzyme activities. As mentioned above, the activities of NER-GO and NER-TR can be measured independently in the same sample by obtaining the signals of A_{570} and A_{410}, respectively. To demonstrate this orthogonal measurement in a vivid way, the enzyme reactions of NERs were performed both individually and in a mixture to obtain the absorbance spectra from 320 nm to 630 nm. The mixture of

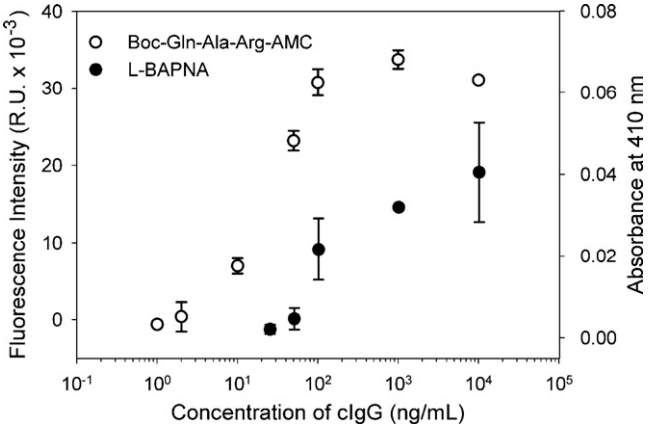

Fig. 4. Correlation between the NER-LISA signal and the target antigen concentration for the detection of chicken IgG (cIgG) using NER-TR/anti-cIgG and MB/anti-cIgG. The white circles represent the fluorogenic signal amplification using Boc–Gln–Ala–Arg–AMC as the substrate, and the black circles represent the chromogenic signal amplification using L-BAPNA as the substrate. The background signal, obtained from no-antigen control reaction, has been subtracted from each value. Error bars indicate the standard deviations in duplicate experiments.

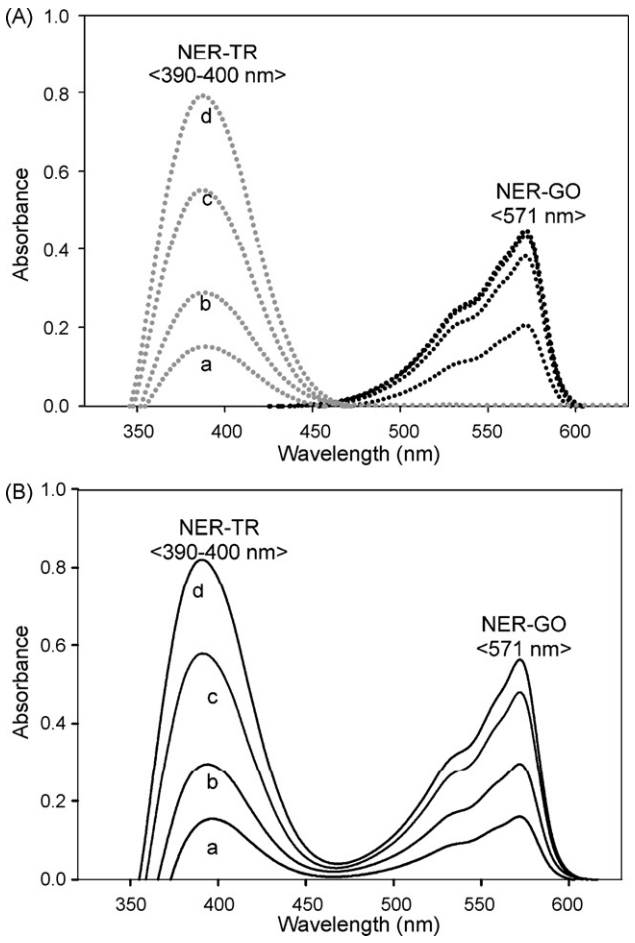

Fig. 5. Time-dependent absorbance spectra with NERs. a-d represent the reaction times of 5, 10, 20 and 30 min, respectively. (A) Spectra of individual NER-GO or NER-TR assays. (B) Spectra of NER-GO and NER-TR assays in the same solution.

NER-GO and NER-TR showed similar GO and TR activities to those from individual measurements (Fig. 5), suggesting that the NER approach effectively prevents the TR's digestion of GO by retaining TR molecules within the mesopores of MCF silica.

To demonstrate the potential use of NER-LISA in simultaneously detecting multi-target antigens, NER-LISA was performed for the detection of hIgG and cIgG by employing NER-GO/anti-hIgG and NER-TR/anti-cIgG, respectively. NER-LISA was performed both individually (single assay) and in a mixture (dual assay), and the detection signals of hIgG and cIgG were obtained from the increase of A_{570} and A_{410}, respectively. As shown in Fig. 6, the detection signals by GO at 570 nm was 0.604 ± 0.105 (17.3% of relative standard deviation, RSD; $n = 3$) and 0.629 ± 0.074 (11.8% of RSD; $n = 3$) in single and dual assays, respectively. On the other hand, the detection signals by TR at 410 nm resulted in 0.040 ± 0.004 (9.5% of RSD; $n = 3$) and 0.040 ± 0.002 (4.6% of RSD; $n = 3$) in single and dual assays, respectively. The similar values either in a single or dual assay suggest that the NER-LISA is applicable to the simultaneous detection of multiple targets with unchanged selectivity.

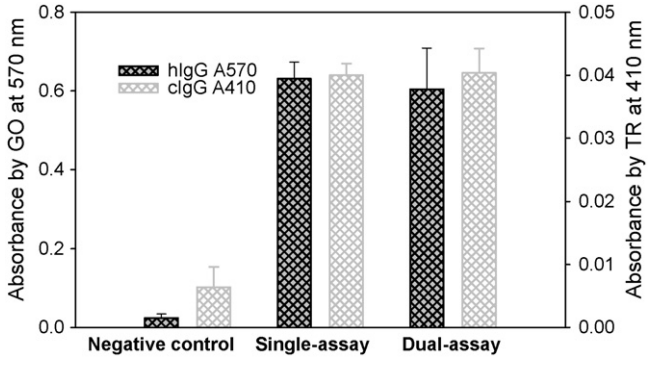

Fig. 6. Simultaneous immunoassay of the human IgG (hIgG) and the chicken IgG (cIgG) using NER-GO/anti-hIgG and NER-TR/anti-cIgG. Negative control represents the assay with none of the target antigens. The single-assay and the dual-assay represent the single-enzyme immunoassay and the simultaneous dual-enzyme immunoassay for the detection of hIgG and cIgG, respectively. In both assays, 10 $\mu g\,mL^{-1}$ each of hIgG and cIgG were used. Error bars indicate the standard deviations in triplicate experiments.

4. Conclusions

We have demonstrated that the NER approach, endowed with high enzyme loading, activity, and stability, can be employed in improving the sensitivity and fidelity of ELISA. Antibody-conjugated NERs of two different enzymes were successfully used for the simultaneous detection of dual target antigens, manifesting the potential of NER-LISA as a multiplexed assay method. This unique approach of NER-LISA has also enabled the use of a protease (trypsin), opening up the door for adopting various proteases and their versatile assays in the ELISA method. For example, the fluorogenic protease substrates can be used for sensitive and orthogonal activity measurements in the NER-LISA approach.

Acknowledgements

This work was supported by the Nano/Bio Science & Technology Program (2005-01291), the Nano Science & Technology Program (M10503000868-08M0300-86810), Pioneer Research Program for Converging Technology (M10711300001-08M1130-00110), the Brain Korea 21 Program and World Class University Program (R31-2008-000-10071-0) of MEST (Ministry of Education, Science and Technology), the Korea Health 21C R&D Project (A04-0041-B21004-08A5-00030B) of MIHWAF (Minister for Health, Welfare, and Family Affairs) and the Korea Foundation for International Cooperation of Science & Technology (KICOS) through a grant (K20601000002-07E0100-00230) provided by the Korean Ministry of Education, Science & Technology (MEST).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.09.005.

References

Ambrosi, A., Castaneda, M.T., Killard, A.J., Smyth, M.R., Alegret, S., Merkoci, A., 2007. Anal. Chem. 79 (14), 5232–5240.
Bradford, M.M., 1976. Anal. Biochem. 72, 248–254.
Fan, J., Shui, W., Yang, P., Wang, X., Xu, Y., Wang, H., Chen, X., Zhao, D., 2005. Chem. Eur. J. 11 (18), 5391–5396.
Hecht, H.J., Kalisz, H.M., Hendle, J., Schmid, R.D., Schomburg, D., 1993. J. Mol. Biol. 229 (1), 153–172.
Hermanson, G.T., 1996. Bioconjugate Techniques. Academic Press, Elsevier, San Diego, pp. 472–476.
Kim, B.C., Lopez-Ferrer, D., Lee, S.M., Ahn, H.K., Nair, S., Kim, S.H., Kim, B.S., Petritis, K., Camp, D.G., Grate, J.W., Smith, R.D., Koo, Y.M., Gu, M.B., Kim, J., 2009. Proteomics 9 (7), 1893–1900.
Kim, E., Lee, B.S., Pyo, H.-B., Song, H.-W., Kim, Y.-P., Choi, I.S., Kim, H.-S., 2008a. Biochip J. 2 (2), 103–110.
Kim, J., Grate, J.W., Wang, P., 2008b. Trends Biotechnol. 26 (11), 639–646.
Kim, J., Jia, H., Wang, P., 2006. Biotechnol. Adv. 24 (3), 296–308.
Kim, J., Lee, J., Na, H.B., Kim, B.C., Youn, J.K., Kwak, J.H., Moon, K., Lee, E., Kim, J., Park, J., Dohnalkova, A., Park, H.G., Gu, M.B., Chang, H.N., Grate, J.W., Hyeon, T., 2005. Small 1 (12), 1203–1207.
Kim, M.I., Kim, J., Lee, J., Jia, H., Na, H.B., Youn, J.K., Kwak, J.H., Dohnalkova, A., Grate, J.W., Wang, P., Hyeon, T., Park, H.G., Chang, H.N., 2007. Biotechnol. Bioeng. 96 (2), 210–218.

Lee, D., Lee, J., Kim, J., Kim, J., Na, H.B., Kim, B., Shin, C.H., Kwak, J.H., Dohnalkova, A., Grate, J.W., Hyeon, T., Kim, H.S., 2005a. Adv. Mater. 17, 2828–2833.
Lee, J., Kim, J., Kim, J., Jia, H., Kim, M.I., Kwak, J.H., Jin, S., Dohnalkova, A., Park, H.G., Chang, H.N., Wang, P., Grate, J.W., Hyeon, T., 2005b. Small 1 (7), 744–753.
Lee, J., Lee, D., Oh, E., Kim, J., Kim, Y.P., Jin, S., Kim, H.S., Hwang, Y., Kwak, J.H., Park, J.G., Shin, C.H., Kim, J., Hyeon, T., 2005c. Angew. Chem. Int. Ed. 44 (45), 7427–7432.
Lee, J.H., Hwang, E.T., Kim, B.C., Lee, S.M., Sang, B.I., Choi, Y.S., Kim, J., Gu, M.B., 2007. Appl. Microbiol. Biotechnol. 75 (6), 1301–1307.
Mano, N., Mao, F., Heller, A., 2003. J. Am. Chem. Soc. 125 (21), 6588–6594.
Minteer, S.D., Liaw, B.Y., Cooney, M.J., 2007. Curr. Opin. Biotechnol. 18 (3), 228–234.
Munge, B., Liu, G., Collins, G., Wang, J., 2005. Anal. Chem. 77 (14), 4662–4666.
Oh, Y.-H., Kim, Y.-P., Kim, H.-S., 2007. Biochip J. 1 (1), 28–34.
Piao, Y., Lee, D., Kim, J., Kim, J., Hyeon, T., Kim, H.-S., 2009. Analyst 134 (5), 926–932.
Schmidt-Winkel, P., Lukens Jr., W.W., Zhao, D., Yang, P., Chmelka, B.F., Stucky, G.D., 1999. J. Am. Chem. Soc. 121, 254–255.
Shui, W., Fan, J., Yang, P., Liu, C., Zhai, J., Lei, J., Yan, Y., Zhao, D., Chen, X., 2006. Anal. Chem. 78 (14), 4811–4819.
Tang, D., Yuan, R., Chai, Y., 2008. Anal. Chem. 80 (5), 1582–1588.
Wang, J., Liu, G., Jan, M.R., 2004. J. Am. Chem. Soc. 126 (10), 3010–3011.
Wang, P., Dai, S., Waezsada, S.D., Tsao, A.Y., Davison, B.H., 2001. Biotechnol. Bioeng. 74 (3), 249–255.
Willner, I., Basnar, B., Willner, B., 2007. FEBS J. 274 (2), 302–309.
Yu, X., Munge, B., Patel, V., Jensen, G., Bhirde, A., Gong, J.D., Kim, S.N., Gillespie, J., Gutkind, J.S., Papadimitrakopoulos, F., Rusling, J.F., 2006. J. Am. Chem. Soc. 128 (34), 11199–11205.
Zuo, C., Yu, W., Zhou, X., Zhao, D., Yang, P., 2006. Rapid Commun. Mass Spectrom. 20 (20), 3139–3144.