Electronic Supplementary Information

Highly Sensitive Immunoassay using Antibodies-Conjugated Spherical Mesoporous Silica with Immobilized Enzymes

Ji Young Eum[a,+], Sang Youn Hwang[a,+], Youngjun Ju[a], Jong Min Shim[b], Yunxian Piao[c], Jinwoo Lee[b], Hak-Sung Kim[c], and Jungbae Kim[a,*]

[a] *Department of Chemical and Biological Engineering, Korea University, Seoul 136-713, Republic of Korea*

[b] *Department of Chemical Engineering, Pohang University of Science and Technology, Pohang, Gyungbuk 790-784, Korea*

[c] *Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea*

+ These authors equally contributed.

Experimental Section

Chemicals and Materials Glucose oxidase (GO), o-dianisidine dihydrochloride (ODS), D-glucose, horseradish peroxidase (HRP), glutaraldehyde (GA, 25%), IgG from human serum (hIgG), anti-human IgG antibody from goat (Fab specific), anti-human IgG antibody from goat (Fc specific), rabbit IgG (rIgG), mouse IgG (mIgG), 3-aminopropyltrimethoxysilane (APTMS), Tween 20, sodium phosphate monobasic, sodium phosphate dibasic, Trizma base, Trizma hydrochloride and phosphate buffered saline (PBS, pH 7.4) were purchased from Sigma-Aldrich (MO, USA). Amplex red was purchased from Invitrogen (OR, USA). Bovine serum albumin (BSA) was purchased from Millipore (IL, USA). The BCA protein assay reagent kit was purchased from Pierce (IL, USA). High-binding 96 well plates were purchased from Corning (NY, USA). Spherical mesoporous silica (S-MPS) was kindly gifted from Fuji Silysia (Aichi, Japan). The particle size of S-MPS is 5.0 ± 0.5 μm and the pore size is 30 ± 3 nm (data from Certificate of Analysis supplied from Fuji Silysia). Coating buffer for capture anti-hIgG antibody (Fab) is 0.1 M carbonate/bicarbonate buffer (pH 9.6). Blocking buffer during immunoassay is 3% BSA (w/v) in PBS (pH 7.4). Washing buffer is 0.05% (v/v) Tween 20 in PBS (pH 7.4) (0.05% PBST). All antibodies and antigens are diluted in washing buffer during immunoassay.

Preparation of amine-functionalized spherical porous silica (amino-SPS) For the preparation of amino-S-MPS, 3-aminopropyltrimethoxysilane (APTMS, 0.5 g, 2.79 mM) was added to 1 g of S-MPS in anhydrous toluene. The mixture was allowed to stir for 24 h at room temperature under argon. The resulting solid was filtered, washed with toluene, and dried under vacuum at room temperature overnight. Morphological investigation was carried out using the scanning electron microscopy (FE-SEM, Hitachi S-4800). The surface area of amino-S-MPS was calculated from the 77 K N_2 adsorption-desorption isotherms using a Tristar II 3020 system (Micromeritics Inc.), based on the Brunauer-Emmett-Teller (BET) method. The pore size distribution was obtained from the adsorption branch using the Barett-Joyner-Halenda (BJH) method. The samples were degassed at 150°C overnight before

measurement. To determine the content of the amines on the silica surfaces, elemental analysis was conducted using Vario EL III.

Preparation of ADS-GO, CA-GO and NER-GO NER-GO was prepared by similar procedure described in elsewhere.[1] In detail, 5 mg of amino-S-MPS was incubated with 1.5 mL of 25 mg/mL GO solution in 0.1 M sodium phosphate buffer (PB, pH 7.0) under 200 rpm shaking for 2 hr. Then, the sample was centrifuged down at 10000 rpm for 10 min, and the supernatant was decanted. After GO adsorption, GA solution (0.5% in 0.1 M PB, pH 7.0) was added and incubated for 3 hr. Then, 0.1 M Tris-HCl buffer (pH 8.0) was added to cap un-reacted aldehyde groups. The sample was excessively washed with 0.1 M PB (pH 7.0), and stored at 4°C before use. As control samples, ADS-GO and CA-GO were prepared. For preparation of simply adsorbed GO in amino-S-MPS (ADS-GO), all procedures for NER-GO were duplicated except the GA treatment step. In other words, 0.1 M PB (pH 7.0) was added instead of GA solution. Covalently attached GO on amino-S-MPS (CA-GO) was prepared by treating amino-S-MPS with GA (0.1% in 0.1 M PB pH 7.0) before the incubation in the GO solution.

Activity and stability of ADS-GO, CA-GO and NER-GO The activities of ADS-GO, CA-GO and NER-GO were measured by a conventional GO assay. 100 μL of each sample was reacted with 890 μL of reaction cocktail (0.17 mM o-dianisidine and 1.72% (w/v) D-glucose) and 10 μL of 600 U/mL of peroxidase solution under shaking (250 rpm). 100 μL of mixture was withdrawn every 3 min for 15 min, and mixed with 900 μL of 0.1 M PB (pH 7.0). The absorbance 500 nm (A500) was measured by using UV spectrophotometer (Shimadzu, UV-2450, Kyoto, Japan). The activity was calculated with absorbance increase at 500 nm per min (A500/min), and further normalized per 1 mg of amino-S-MPS. The stabilities of ADS-GO, CA-GO and NER-GO were checked under continuous shaking (200 rpm) at room temperature for 26 days. The stabilities of the samples were checked by measuring the residual enzyme activity of each sample at the specific time point, and the relative activity was calculated from the ratio of residual activity to the initial activity of each sample.

Preparation of antibody-conjugated GO For preparation of antibody-conjugated GO, oxidized GO was conjugated with anti-hIgG antibody by reductive amination coupling.[2] Briefly, 10 mg of GO was dissolved with 5 mg of sodium meta-periodate in 1 mL of 0.1 M PB (pH 7.0), and incubated in the dark at room temperature for 30 min. The sample was purified using desalting column (PD-10, GE Healthcare, USA) by using 0.1 M PB (pH 7.0) as an elution buffer. After desalting, the sample was concentrated to 5.0 mg/mL of GO by using a centrifugal filter (Centricon YM 100, Millipore, USA) by exchanging the buffer to 0.2 M sodium bicarbonate buffer (pH 9.6). The oxidized GO was mixed with anti-hIgG antibody (Fc specific, 1 mg/mL in 0.2 M sodium bicarbonate buffer, pH 9.6) in a 2:1 molar ratio of GO to anti-hIgG antibody, and incubated in a dark for 2 hr. 5.0 M sodium cyanoborohydride (50 mM as a final concentration) in 1 M NaOH was added to the mixture, and reacted in a dark for 30 min. The reaction was stopped and blocked by adding 1.0 M ethanolamine hydrochloride solution (pH 9.6). Finally, GO-conjugate antibody was purified using a desalting column (PD-10) with 0.01 M PBS (pH 7.4), and concentrated with the centrifugal filter (Centricon YM 100). The final concentration of GO-conjugated antibody was adjusted to 5 mg/mL and stored at -20°C before use.

Preparation of antibody-conjugated NER-GO For preparation of antibody-conjugated NER-GO, NER-GO was prepared without capping with 0.1 M Tris-HCl (pH 8.0). Firstly, non-Tris capped NER-GO (1 mg/mL) was incubated with 0.1% BSA (w/v) in 0.01 M PBS (pH 7.4) for 30 min. After the incubation, non-reacted BSA was removed by centrifuge (10000 rpm, 5 min). The BSA coated NER-GO was incubated in 0.05% GA solution at room temperature for 10 min. The GA solution was removed after centrifugation (1000 rpm, 5 min), and the sample was washed 3 times with 0.1 M PB (pH 7.0). GA treated NER-GO was incubated in 0.2 mg/mL anti-hIgG antibody (Fc specific, in 0.01 M PBS, pH 7.4) at room temperature for 2 h. Unbound antibodies were washed away with 0.01 M PBS (pH 7.4) after centrifugation (10000 rpm, 5 min). The concentration of unbound antibodies was measured by the BCA assay to calculate the concentration bound antibodies on NER-GO. After

antibodies immobilization, 0.1 M Tris-HCl (pH 8.0) was added to cap unreacted aldehyde groups at room temperature for 1 h, and the final sample was stored at 4°C in PBS with 0.1% BSA (w/v).

Immunoassay using antibody-conjugated NER-GO and antibody-conjugated GO The sandwich immunoassay was performed by using antibody-conjugated NER-GO and antibody-conjugated GO for the detection of hIgG as a model analyte. 25 μL of anti-hIgG antibody solution (Fab specific, 10 μg/mL in coating buffer) was added to each well, and incubated at 4°C for overnight. After decanting the unbound antibody solution, wells were blocked by addition of 100 μL of blocking buffer and incubated at room temperature for 1 h. After blocking buffer was removed, it was washed 5 times with 150 μL of washing buffer. 50 μL of hIgG solutions (0 to 10 μg/mL in 0.05% PBST) were added to each well and incubated at 37°C for 1 h. After the incubation, unreacted hIgG solutions were removed, and 5-time washings were done with 150 μL of washing buffer. 50 μL of antibody-conjugated NER-GO (100 μg/mL in 0.05% PBST) were added to each well, and incubated at 37°C for 30 min. Unbound antibody-conjugated NER-GO was excessively washed with 150 μL of washing buffer. After washing, 50 μL of substrate solution (100 μM Amplex red, 0.2 U/mL HRP, and 100 mM glucose in 50 mM PB (pH 7.4)) was added in each well, and the absorbance was measured at 570 nm every 5 min under shaking. As a control experiment, antibody-conjugated GO was used instead of antibody-conjugated NER-GO. The input antibody concentration to each well was controlled as the same in both antibody-conjugated GO and antibody-conjugated NER-GO (1 μg/mL per well). To check the specificity of hIgG detection by using antibody-conjugated NER-GO, 1 μg/mL of rIgG or mIgG were used as a negative control. Every experiment was performed in triplicates, and errors were calculated by the standard deviation. The limit of detection (LOD) was estimated by the hIgG concentration, which produces a signal greater than the sum of the averaged blank signal and three times their standard deviation. The coefficient of variations (CV) was calculated by the percentage ratio of standard deviation to the averaged signal.

Supporting Figures and Tables

Table S1. Comparison of various ELISA approaches for hIgG detection.

Enzyme reaction time	ELISA type	LOD (ng/mL)	Dynamic range (ng/mL)	Ref.
5 min	NER-LISA Spherical mesoporous silica (S-MPS)	0.5	0.1-1000	This work
	Conventional ELISA	10	10-1000	This work
30 min	NER-LISA Spherical mesoporous silica (S-MPS)	0.5	0.1-100	This work
	Conventional ELISA	10	10-500	This work
	NER-LISA Amorphous mesoporous silica	10	10-10000	(1)

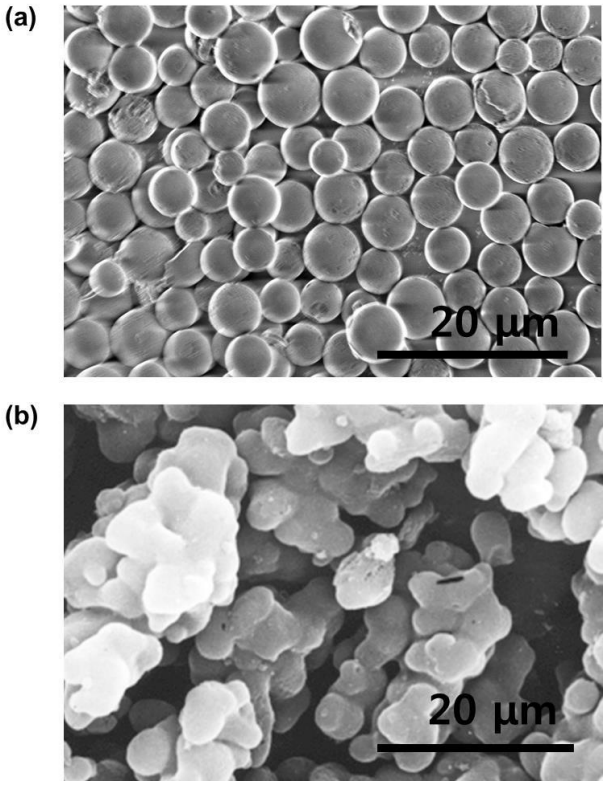

Figure S1. The SEM images of S-MPS (a) and amorphous silica (b).

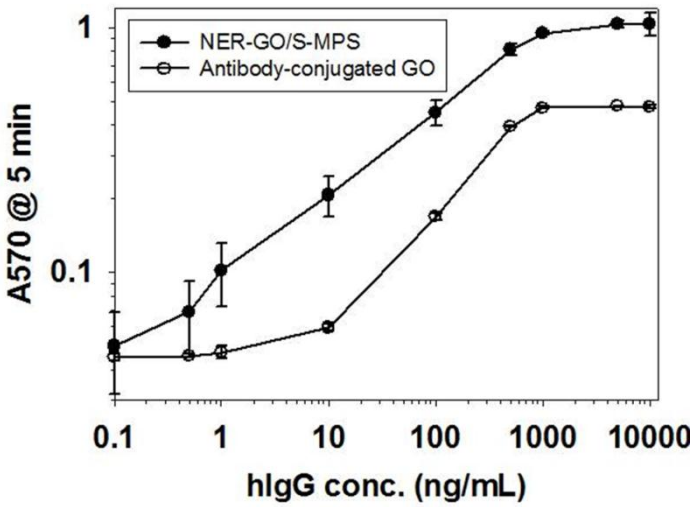

Figure S2. Calibration curves in the log-log scale for the determination of dynamic ranges of hIgG detection in conventional ELISA and NER-LISA.

References

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2. G. T. Hermanson, ed., *Bioconjugate techniques*, Academic Press San Diego, 1996.