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Bionanocapsule-based enzyme–antibody conjugates for enzyme-linked immunosorbent assay

Masumi Iijima [a,b], Takashi Matsuzaki [a], Hiroyasu Kadoya [a], Satoko Hatahira [c], Shingo Hiramatsu [c], Giman Jung [c], Katsuyuki Tanizawa [a,*], Shun'ichi Kuroda [a,b,*]

[a] Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka 567-0047, Japan
[b] Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan
[c] New Frontiers Research Laboratories, Toray Industries, Kamakura, Kanagawa 248-8555, Japan

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ABSTRACT

Macromolecules that can assemble a large number of enzyme and antibody molecules have been used frequently for improvement of sensitivities in enzyme-linked immunosorbent assays (ELISAs). We generated bionanocapsules (BNCs) of approximately 30 nm displaying immunoglobulin G (IgG) Fc-binding ZZ domains derived from *Staphylococcus aureus* protein A (designated as ZZ-BNC). In the conventional ELISA using primary antibody and horseradish peroxidase-labeled secondary antibody for detecting antigen on the solid phase, ZZ-BNCs in the aqueous phase gave an approximately 10-fold higher signal. In Western blot analysis, the mixture of ZZ-BNCs with secondary antibody gave an approximately 50-fold higher signal than that without ZZ-BNCs. These results suggest that a large number of secondary antibody molecules are immobilized on the surface of ZZ-BNCs and attached to antigen, leading to the significant enhancement of sensitivity. In combination with the avidin–biotin complex system, biotinylated ZZ-BNCs showed more significant signal enhancement in ELISA and Western blot analysis. Thus, ZZ-BNC is expected to increase the performance of various conventional immunoassays.

Enzyme immunoassay (EIA),[1] radioimmunoassay (RIA), and fluoroimmunoassay (FIA) have been widely used for high-throughput screening of immunocomplexes. To increase the sensitivity of these immunoassays, two types of macromolecules have been used in the reaction with antibody. One type of macromolecule allows clustering of antibodies and labeling molecules (e.g., enzyme, radioactive material, fluorescence). For example, polymeric horseradish peroxidase (HRP)–streptavidin conjugate [1], 3DNA dendrimer [2], and immunoglobulin G (IgG)–poly-D-glutamic acid–$(HRP)_n$ conjugate [3] have been used previously. However, these macromolecules require chemical modification of antibodies and do not allow the oriented immobilization of antibodies [4] that improves the avidity and antigen recognition of antibodies [5]. In general, chemical modification is considered to reduce the stability or antigen-binding activity of antibodies. Another type of macromolecule is expected to permit the oriented immobilization of antibodies. For example, nanoparticles have been used for displaying antibodies on their surface (e.g., streptavidin-conjugated nanobeads [6], biotin-coated liposomes [7]). Because biotinylation occurred randomly at free amino groups on the surface of antibodies, these nanoparticles partially accomplished oriented immobilization of antibodies using a avidin–biotin complex (ABC). These results encouraged us to develop macromolecules that can assemble antibodies and labeling molecules without chemical modification in the manner of oriented immobilization.

On the other hand, we previously developed a yeast-derived hollow nanoparticle applicable for pinpoint delivery of drugs and genes [8]. The nanoparticle, bionanocapsule (BNC), has a diameter of approximately 30 nm and is composed of hepatitis B virus (HBV) surface antigen (HBsAg) L proteins embedded in a liposome [9]. The L protein is a three-membrane-spanning protein possessing a pre-S region at the N terminal of the S region (see Fig. 1A) [10]. BNCs can incorporate various therapeutic materials (e.g., drugs, genes) by electroporation [8] and liposome fusion [11] and can deliver them specifically to the human liver [8] based on the liver-specific recognition ability of the pre-S region [12]. Recently, to alter the tissue specificity of BNC, our collaborators made a

* Corresponding authors. Fax: +81 6 6879 8460 (K. Tanizawa), +81 52 789 5227 (S. Kuroda).
E-mail addresses: tanizawa@sanken.osaka-u.ac.jp (K. Tanizawa), skuroda@agr.nagoya-u.ac.jp (S. Kuroda).

[1] *Abbreviations used:* EIA, enzyme immunoassay; RIA, radioimmunoassay; FIA, fluoroimmunoassay; HRP, horseradish peroxidase; IgG, immunoglobulin G; ABC, avidin–biotin complex; BNC, bionanocapsule; HBV, hepatitis B virus; HBsAg, HBV surface antigen; ZZ-BNC, IgG Fc-interacting region (Z domain) derived from *Staphylococcus aureus* protein A; ELISA, enzyme-linked immunosorbent assay; TEM, transmission electron microscopy; OVA, ovalbumin; PBS, phosphate-buffered saline; TMB, 3,3′,5,5′-tetramethylbenzidine; IgE, immunoglobulin E; PVDF, polyvinylidene fluoride; EGFR, epidermal growth factor receptor; EGFP, enhanced green fluorescent protein; LOD, limit of detection; SD, standard deviations; LOQ, limit of quantitation.

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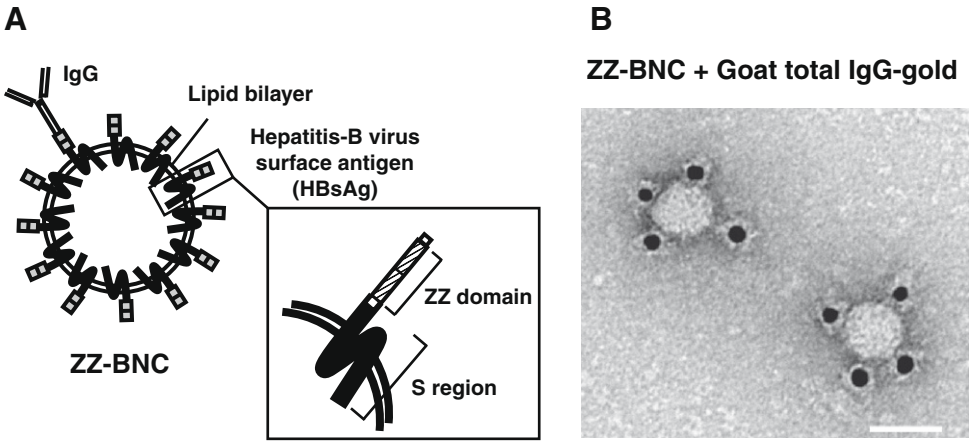

Fig. 1. (A) Schematic structure of a ZZ-BNC. (B) Transmission electron microscopy (TEM) images of ZZ-BNC conjugated with 10-nm gold particle-labeled goat total IgG. Bar = 40 nm.

derivative of BNC by replacing the pre-S region with a tandem sequence of the IgG Fc-interacting region (Z domain) derived from *Staphylococcus aureus* protein A [13] and designated it ZZ-BNC [14] (Fig. 1A). ZZ-BNC allowed us to display antibodies on its surface and to deliver various therapeutic materials to tissues of interest in an antibody-dependent manner [14].

These properties led us to imagine that ZZ-BNC spontaneously adsorbs antibodies in the manner of oriented immobilization. In this study, we examined whether ZZ-BNCs could contribute to signal enhancement of enzyme-linked immunosorbent assay (ELISA) through formation of IgG–ZZ-BNC complexes.

Materials and methods

ZZ-BNCs

ZZ-BNCs were overexpressed in *Saccharomyces cerevisiae* AH22R$^-$ carrying the ZZ-BNC expression plasmid pGLD-ZZ50 [14]. According to the preparation method for BNCs [8], ZZ-BNCs were extracted by the disruption with glass beads and purified using an AKTA chromatography system (GE Healthcare, Amersham, UK) by affinity chromatography on porcine IgG and gel filtration.

Transmission electron microscopy

ZZ-BNCs (1 μg as protein) were mixed with 10-nm gold particle-labeled goat total IgG (50 μl, Sigma–Aldrich, St. Louis, MO, USA), adsorbed onto a carbon-coated copper grid (JEOL, Tokyo, Japan), negatively stained using 2% (w/v) phosphotungstic acid (pH 7.0), and subjected to transmission electron microscopy (TEM) using a model JEM1011 (Jeol).

ELISA for ovalbumin on solid phase

Ovalbumin (OVA, 100 μl, 0–6.25 ng/ml, Sigma–Aldrich) was adsorbed to each well of a Nunc–Immuno Plate II (96 wells, Nalge Nunc International, Rochester, NY, USA). The plate was kept at 4 °C overnight and washed three times with PBST (200 μl of phosphate-buffered saline [PBS], 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, and 2 mM KH_2PO_4 [pH 7.4] containing 0.05% [v/v] Tween 20). Antibodies were diluted with PBST containing 5% (w/v) skimmed milk (Nacalai Tesque, Kyoto, Japan). A primary antibody, a mouse anti-OVA IgG_1 antibody (100 μl, 0.4 μg/ml, Abcam, Cambridge, UK), was added to each well, incubated at room temperature for 1.5 h, and washed three times with PBST. A secondary antibody, HRP-conjugated rabbit anti-mouse IgG (100 μl, 2 μg/ml, Sigma–Aldrich), was added to each well, incubated at room temperature for 1.5 h, and washed three times with PBST. When biotinylated rabbit anti-mouse IgG (100 μl, 2 μg/ml, Sigma–Aldrich) was used as the secondary antibody, the ABC system (ABC peroxidase staining kit, Pierce, Rockford, IL, USA) was used for the labeling with HRP. The colorimetric reaction was carried out at room temperature for 15 min in 100 μl per well of a 3,3′,5,5′-tetramethylbenzidine (TMB) substrate kit (Pierce). The reaction was stopped with 100 μl of 2 N H_2SO_4. Absorbance at 450 nm was measured on a Varioskan microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) using absorbance at 690 nm as the reference. When the calibration curve was generated using 0–6.25 ng/ml OVA ($n = 8$), the wells containing 0 ng/ml OVA were defined as blank wells for subtracting background.

ELISA for anti-OVA immunoglobulin E antibodies in aqueous phase

OVA (100 μl, 15 μg/ml) was adsorbed onto each well of a Nunc–Immuno Plate II. The plate was kept at 4 °C overnight and washed three times with 200 μl of PBST. Mouse anti-OVA immunoglobulin E (IgE) antibody (0–6.25 ng/ml, 100 μl, AbD Serotec, Oxford, UK) diluted with PBST containing 5% skimmed milk was added to each well, incubated at room temperature for 1.5 h, and washed three times with PBST. An HRP-conjugated rabbit anti-mouse IgE Fc antibody (100 μl, 5 μg/ml, Nordic Immunological Laboratories, Tilburg, Netherlands) was added to each well, incubated at room temperature for 1.5 h, and washed three times with PBST. When biotin-labeled rabbit anti-mouse IgE Fc antibody (100 μl, 5 μg/ml, Nordic Immunological Laboratories) was used as the secondary antibody, the ABC peroxidase staining kit was used for the labeling with HRP. The colorimetric reaction was carried out at room temperature for 15 min in 100 μl per well of a TMB substrate kit. The reaction was stopped with 100 μl of 2 N H_2SO_4. Absorbance at 450 nm was measured on a Varioskan microplate reader using absorbance at 690 nm as the reference. When the calibration curve was generated using 0–6.25 ng/ml anti-OVA IgE ($n = 8$), the wells containing 0 ng/ml anti-OVA IgE were defined as blank wells for subtracting background.

Western blot analysis

Here 1 μl of OVA (0.001–10 mg/ml) was blotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA,