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Fluorophore-labeled nanocapsules displaying IgG Fc-binding domains for the simultaneous detection of multiple antigens

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ABSTRACT

Simultaneous detection of multiple antigens by conventional immunological methods has been limited by the source of primary antibodies. Each antibody should be derived from a different host species (or subclass of immunoglobulin (Ig)) for suppressing the cross-reactions of secondary antibodies. Here we describe an innovative method for simultaneous, rapid, and sensitive detection of multiple antigens using ~30-nm bio-nanocapsules (BNCs) displaying IgG Fc-binding Z domains derived from *Staphylococcus aureus* protein A (ZZ-BNC). When Cy2-labeled ZZ-BNC (Cy2-ZZ-BNC) was used instead of Cy2-labeled secondary antibody in western blot analysis, both sensitivity and signal intensity were significantly increased. The complex of Cy5-ZZ-BNC and mouse IgG2a (which shows moderate affinity to the Z domain) was not dissociated, even in the presence of 8-fold excess of free mouse IgG2a. In addition, crosslinking with BS^3 (bis-sulfosuccinimidyl suberate) efficiently stabilized the interaction. The ZZ-BNCs labeled with various Cy dyes facilitated the simultaneous detection of multiple antigens using primary antibodies derived from the same host species, by western blot analysis, immunocytochemistry and flow cytometry, which could expand the possibility of bio-imaging probes in various immunofluorescence techniques.

1. Introduction

The simultaneous detection of multiple antigens in one specimen by immunological methods (e.g., western blot analysis, immunocytochemistry, flow cytometric analysis, and immunohistochemistry) has been considered as a powerful tool for the elucidation of functions and cellular localizations of various biomolecules. However, primary antibodies derived from the same host species cannot be used simultaneously, because one secondary antibody may cross-react with multiple primary antibodies. Usually, we have chosen primary antibodies from different or non-crossreactive species/subclasses [1]. Such convenient pairs of antibodies are not always available. Even if we have excellent primary antibodies, the cross-reactivity limitation has hampered the expansion of possibilities of these immunological methods. Generally, 'sequential immunolabeling methods' have been applied to enable the detection of multiple antigens with primary antibodies from the same host species. Each immunolabeling step is carried out using a secondary antibody labeled with a distinct fluorophore [1], enzyme, or hapten [2], which are sometimes coupled by heat treatment [3,4], low pH, and detergent [5] to eliminate the residual antibodies on the specimen for subsequent immunolabeling steps. Tyramide signal amplification (TSA) [6] is also used to reduce the amount of primary antibodies in the first immunolabeling step. Alternatively, each primary antibody used in the first immunolabeling step is blocked by anti-IgG Fab fragments to reduce the cross-reaction of secondary antibodies in the second immunolabeling step [7,8]. Fluorophore-labeled anti-IgG Fab fragments [9], enzyme-labeled anti-IgG Fab/F(ab′)$_2$ fragments [10], and fluorophore-labeled anti-IgG F(ab′)$_2$ fragments [11] could be used for the immunolabeling of primary antibodies in the first step. However, these methods are time-consuming, not applicable for the simultaneous immunolabeling of multiple antigens, and practically limited to two immunolabelings. False-positive signals may also occur by the cross-reaction of secondary antibodies. To overcome these problems, 'simultaneous immunolabeling methods' have been developed to detect multiple antigens with primary antibodies from the same host species as follows; (a) direct labeling, where each primary antibody is directly labeled with a distinct enzyme [12] or fluorophore [13]; (b) protein A complex,

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where each primary antibody is conjugated with fluorophore-labeled *Staphylococcus aureus* protein A [14]; and (c) anti-IgG or anti-Fc Fab fragment, where each primary antibody is conjugated with a fluorophore-labeled anti-Fc or anti-IgG Fab fragment [15,16]. These methods could facilitate the simultaneous immunolabeling for multiple antigens in one step. However, in the case of 'direct labeling method,' the chemical modifications of each primary antibody are laborious and require a large amount of antibodies, which may damage the antigen-binding activity and stability of primary antibodies. The 'protein A method' is applicable for a variety of IgGs, but it does not sufficiently exclude the possibility that primary antibodies on protein A are replaced by other primary antibodies, which may increase the cross-reactions of the primary antibodies. Additionally, the amount of fluorophore per one molecule of primary antibody cannot be increased significantly over that of conventional methods. The 'anti-IgG or anti-Fc Fab fragment method' requires the preparation of Fab fragments for each animal species and IgG subclass, which is time-consuming and laborious. The signal intensity is also comparable to that of conventional methods [15,17].

We have previously generated a nanocapsule of ~30 nm diameter by expressing the hepatitis B virus surface antigen (HBsAg) L gene in *Saccharomyces cerevisiae* [18]. The nanoparticle (abbreviated later as BNC, bio-nanocapsule) is composed of about 110 molecules of HBsAg L proteins embedded in a liposome. Our collaborators have recently made a derivative of BNC in which the N-terminal region (amino acid residue from 51 to 159) of L protein is replaced with a tandem sequence of the IgG Fc-interacting region (Z domain) derived from protein A [19] and designated it ZZ-BNC (Fig. 1) [20]. ZZ-BNC displays about 120 molecules of the ZZ-L protein (N-terminally ZZ-fused L protein) on its surface, and can capture ~60 mouse total IgG molecules, as well as displaying all the IgG Fv regions outwardly for effective antigen binding [21]. When ZZ-BNCs were used as a scaffold of antibodies for the immunosensor chip of quartz crystal microbalance (QCM) and surface plasmon resonance (SPR), they markedly improved the sensitivity, antigen-binding capacity, and affinity of the antibodies on the gold surface of immunosensor chip, presumably by the oriented immobilization of the antibodies. Furthermore, in conventional enzyme-linked immunosorbent assays (ELISAs) and western blot analyses, the addition of ZZ-BNCs with secondary antibodies in the aqueous phase enhanced the sensitivities of antigen detection by 10-fold and 50-fold, respectively [22]. These results indicated that ZZ-BNC contributes not only to the clustering of antibodies and labeling molecules, but also to the oriented immobilization of the antibodies.

In this paper we present a study of the capacity of fluorophore-labeled ZZ-BNCs to enhance the sensitivity and signal intensity of various immunological assays and the possibility of establishing simultaneous immunolabeling methods for multiple antigens.

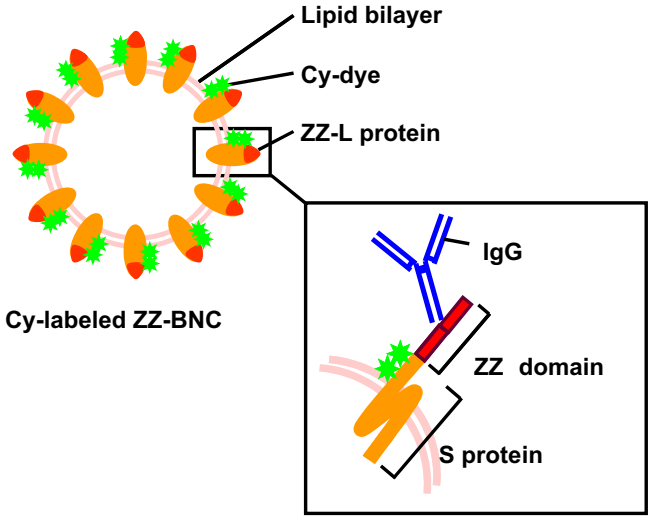

Fig. 1. Capsular structure of Cy-labeled ZZ-BNC. One ZZ-BNC particle consists of ~120 ZZ-L proteins and a lipid bilayer.

2. Materials and methods

2.1. BNCs

ZZ-BNCs were overexpressed in *S. cerevisiae* AH22R$^-$ cells carrying the ZZ-BNC-expression plasmid pGLD-ZZ50 [18,20]. According to the preparation method for BNC [23,24], ZZ-BNCs were extracted by the disruption with glass beads and purified by affinity chromatography on porcine IgG and gel filtration equipped on an AKTA system (GE Healthcare, Amersham, UK).

2.2. Reagents

Cy2-, Cy3-, Cy5-, and Cy7-bis-reactive Dye were from GE Healthcare. Rabbit muscle actin was from Sigma–Aldrich Inc. (Saint Louis, MO, USA). Recombinant human desmin and recombinant human vimentin were from Progen Biotechnik GmbH (Heidelberg, Germany). Tubulin from porcine brain was from Cytoskeleton Inc. (Denver, CO, USA). Cy2- or Cy5-labeled goat-derived anti-mouse IgG were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Zenon Cy2 mouse IgG2a labeling kit was from Molecular Probes, Inc. (Eugene, OR, USA). The primary antibodies used in this study are listed in Table 1. Glutathione-*S* transferase (GST) was expressed in *Escherichia coli* BL21 carrying the GST-expression plasmid pGEX6P-1 (GE Healthcare), and was purified by affinity chromatography on glutathione (GE Healthcare).

2.3. Conventional western blot analysis

Each antigen (actin, desmin, GST, vimentin; 0.5–500 ng) was separated by a 0.1% (w/v) sodium dodecyl sulfate-12.5% (w/v) polyacrylamide gel (12.5% SDS-PAGE), and blotted onto an Immobilon-FL polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% (w/v) skimmed milk (Nacalai Tesque, Kyoto, Japan) in TBST (20 mM Tris–HCl, 140 mM NaCl, 0.05% (v/v) Tween-20, pH 7.4) at room temperature for 30 min, and then incubated at room temperature for 1 h with each primary antibody (anti-actin mouse IgG2a, anti-desmin mouse IgG2a, anti-GST mouse IgG2a, and anti-vimentin mouse IgG2a; 1 μg/mL). These membranes were washed three times with TBST, and incubated with the Cy2-labeled goat-derived anti-mouse IgG (2 μg/mL) secondary antibody at room temperature for 1 h. After washing three times with TBST, the Cy2-derived fluorescence (emission 506 nm) was visualized under a Typhoon FLA-9000

Table 1
Primary antibodies used in this study.

Antigen	Species	Manufacturer[a]	Binding affinity to ZZ-BNC (%)[b]
Actin	Mouse IgG2a	Sigma–Aldrich	29
Desmin	Mouse IgG2a	Abnova	29
GST	Mouse IgG2a	Nacalai Tesque	29
Vimentin	Mouse IgG2a	Progen	29
β-tubulin	Mouse IgG2b	Millipore	31
Tom20	Mouse IgG2a	Santa Cruz	29
LAMP2	Mouse IgG2a	Santa Cruz	29
PML	Mouse IgG1	Santa Cruz	6
EGFR	Mouse IgG2a	Katayama	29
Integrin β1	Mouse IgG2a	Santa Cruz	29
	Mouse total IgG	Sigma–Aldrich	100
	Human total IgG	Sigma–Aldrich	80
	Rat total IgG	Sigma–Aldrich	10
	Mouse IgG1	Sigma–Aldrich	6
	Mouse IgG2a	Sigma–Aldrich	29
	Mouse IgG2b	Sigma–Aldrich	31

[a] Sigma–Aldrich, Inc. (Saint Louis, MO, USA); Abnova Corporation (Taipei, Taiwan); Nacalai Tesque, Inc. (Kyoto, Japan); Progen Biotechnik GmbH (Heidelberg, Germany); Millipore Corporation (Bedford, MA, USA); Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); Katayama Chemical Industries Co., Ltd. (Osaka, Japan).
[b] Binding affinity to each antibody was defined as percentage (%) of that of ZZ-BNC to mouse total IgG [21].