Protein Binders Specific for Immunoglobulin G from Different Species for Immunoassays and Multiplex Imaging

Sukyo Jeong,†,§ Woosung Heu,†,§ Jong-won Kim,‡ and Hak-Sung Kim*†

†Department of Biological Sciences, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 305-701, Korea
‡Graduate School of Nanoscience and Technology, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 305-701, Korea

Supporting Information

ABSTRACT: An immunoassay is the most widely used method for analyzing a variety of analytes based on antigen−antibody interactions in the biological and medical sciences. However, the use of secondary antibodies has certain shortcomings, such as a high cost, cross-reactivity, and loss of binding affinity during labeling. Herein, we present the development of repebodies specifically binding to immunoglobulin G with a different origin, which is a small-sized nonantibody scaffold composed of leucine-rich repeat (LRR) modules, for use in immunoassays and imaging. Repebodies specific for IgG from different species (i.e., mouse, human, and rabbit) were selected through a phage display, and their affinities were matured using a modular engineering approach. The respective repebodies were labeled with various signal generators such as horseradish peroxidase (HRP), a fluorescent dye, and quantum dots, and the resulting repebodies were used as alternatives to conventional secondary antibodies in typical immunoassays and imaging. The labeled repebodies enabled the detection of diverse target analytes with high sensitivity and specificity, showing a negligible cross-reactivity. Moreover, the repebodies labeled with different color-emitting quantum dots allowed the imaging of cell-surface receptors and proteins in a multiplex manner. The developed repebodies can be effectively used for sensitive immunoassays and multiplex imaging.

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and DNA aptamers were selected for Fc region of IgG using a SELEX and were shown to have a binding capability for IgGs with a different species.\textsuperscript{15,14}

Herein, we present the development of repebodies specific for immunoglobulin G with a different origin as alternatives to secondary antibodies for immunoassays and multiplex imaging. We previously developed a repebody scaffold composed of leucine-rich repeat (LRR) modules.\textsuperscript{16,27} The repebody scaffold was shown to be highly stable over a wide range of pH and temperature values and is overexpressed in bacteria. We selected repebodies specific for IgGs from different species (namely, human, mouse, and rabbit) through a phage display. The selected repebodies were subjected to affinity maturation through a modular evolution approach. Signal generators, such as HRP, and fluorescent dye and quantum dots (QDs), were conjugated to their respective repebodies, which were used for an enzyme-linked immunosorbent assay (ELISA), a Western blot analysis, and the multiplex imaging of a variety of targets. The utility and potential of the repebodies were corroborated through a sensitive analysis and multiplex imaging of diverse targets. The details are reported herein.

\section*{MATERIALS AND METHODS}

\textbf{Construction of a Repebody Library and Phage Display Selection.} A repebody library was constructed by randomizing either four variable sites on each LRRV1 and LRR1 module or the C-terminal region (residues from 240 to 245) using degenerate codons as described in our previous work.\textsuperscript{16} The DNA fragments of the repebodies were cut using EcoRI and XhoI, cloned into a pBEB118N vector, and transformed into \textit{Escherichia coli} (\textit{E. coli}) strain XL1-blue (Stratagene) through electroporation. Repebodies specifically binding to mouse and rabbit IgG were selected through a phage display as described in our previous work.\textsuperscript{16} Briefly, immunotubes (Greiner) were coated with 30 μg of mouse (Calbiochem, no. 401122) or rabbit IgG (Sigma-Aldrich, no. I5006) in a phosphate-buffered saline (PBS, pH 7.4) overnight at 4°C. The tubes were treated with 1% BSA in phosphate-buffered saline Tween-20 (PBST), followed by incubation with 1 mL of a phage solution (1.0 × 10^12 cfu/mL) for 2 h at room temperature. After removing the phage solution, the immunotubes were washed five times with 5 mL of PBST, and the target-bound phages were eluted using a 0.2 M glycine-HCl solution (pH 2.2) for 12 min at room temperature. XL1-Blue F\textsuperscript{'} \textit{E. coli} cells were infected with the eluted phages and incubated for 30 min at 37°C, followed by further incubation for 30 min at 37°C while shaking at 200 rpm. The infected cells were spread onto a 2xYT plate containing 50 μg/mL of ampicillin, 25 μg/mL of tetracycline, and 1% glucose (2xYT/ATG), followed by incubation at 30°C overnight while shaking at 250 rpm. The harvested cells were further grown in 2xYT/ATG until the absorbance at 600 nm reached 0.4–0.7. The cells were superinfected with a VCS M13 helper phage (Stratagene) and grown in a fresh 2xYT medium containing 50 μg/mL of ampicillin, 50 μg/mL of kanamycin, and 25 μg/mL of tetracycline (2xYT/ATG) with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30°C for 16 h. Following centrifugation of the cells, the phages in the solution were incubated with 20% PEG/NaCl at 4°C and precipitated through centrifugation at 10,000g for 1 h. The precipitated phages were suspended in 1 mL of PBS and centrifuged at 13,000 rpm for 10 min to remove the PEG. After five rounds of biopanning, each colony was seeded in a 96-deep well plate (Nunc) containing 150 μL of 2xYT/ATG and cultured overnight at 37°C while shaking at 200 rpm for a phage ELISA. The cells were inoculated in 150 μL of 2xYT/ATG, cultured for 6 h, and infected with an M13 helper phage. A volume of 150 μL of a fresh 2xYT/ATG medium was added to each well and incubated at 30°C overnight while shaking at 250 rpm.

Mouse IgG\textsubscript{1} or rabbit IgG (0.5 μg/well) were coated onto a 96-well Maxisorp plate (Nunc) and incubated at 4°C overnight. Following a blocking with 1% BSA in PBST (blocking buffer) at room temperature for 1 h, the purified phages from the supernatant were added to mouse IgG\textsubscript{1} or rabbit IgG (0.5 μg/well) coated onto a 96-well Maxisorp plate (Nunc) and incubated for 2 h at room temperature. The plates were washed with PBST three times and incubated with HRP-conjugated anti-M13 monoclonal antibody (GE Healthcare, no. 27942101) at 1:5000 dilutions in blocking buffer for 1 h. After a washing with PBST five times, 100 μL of tetramethylbenzidine (TMB) (Sigma-Aldrich) was added to each well to generate a signal. A stop solution of 100 μL of 1 N H\textsubscript{2}SO\textsubscript{4} was added after 10 min. The signals were scanned using an Infinite N200 plate reader (Tecan) at 450 nm. For affinity maturation of the selected repebodies, six additional variable sites at a nearby module were randomized and subjected to phage display selection according to the above procedure.

\textbf{Expression and Purification of Repebodies.} The genes encoding the selected repebodies were inserted into NdeI and XhoI restriction sites of a pET21a vector (Invitrogen) and transformed into \textit{E. coli} DH5α cells by heat shock (42°C, 90 s). The recombinant plasmid was transformed into \textit{E. coli} Origami-B cells (Merck Bioscience), and the colony was inoculated into an Luria–Bertani (LB) medium with 50 μg/mL of carbenicillin, 50 μg/mL of kanamycin, and 25 μg/mL of tetracycline at 37°C until the OD\textsubscript{600} reached approximately 0.5–0.7. The cells were induced with 0.5 mM IPTG at 18°C by shaking at 200 rpm for 20 h and harvested through centrifugation at 5000g for 10 min. The collected cells were suspended using a lysis buffer (50 mM Na\textsubscript{2}HPO\textsubscript{4}, 300 mM NaCl, 10 mM imidazole, at pH 8.0) and sonicated. Cell debris was removed through centrifugation at 16,000 rpm for 1 h at 4°C. The supernatant was collected and subjected to purification using a Ni-NTA Superflow (Qiagen). Histidine-tagged proteins were loaded into a column packed with a Ni-NTA resin, followed by washing using a washing buffer (50 mM Na\textsubscript{2}HPO\textsubscript{4}, 300 mM NaCl, 20 mM imidazole, at pH 8.0) until the proteins could no longer be detected through a Bradford assay. The proteins were eluted using an elution buffer (50 mM Na\textsubscript{2}HPO\textsubscript{4}, 300 mM NaCl, 250 mM imidazole, at pH 8.0), and the eluted proteins were further purified using gel permeation chromatography (Superdex 75, GE Healthcare) with a buffer change to PBS.

\textbf{Isothermal Titration Calorimetry (ITC).} A microcal iTC200 (Malvern) was used to determine the binding affinity of the repebodies to mouse and rabbit IgG at 25°C. As a general procedure, a 0.2 mM repebody was titrated with 0.02 mM IgG through a total of 20 injections. The values of ΔH were determined by fitting the integrated exothermal peaks using the Origin program (OriginLab).

\textbf{Labeling of Repebodies with Fluorescent Dye, HRP, and Quantum Dots.} A fluorescein-labeled repebody was obtained by mixing 1 mg of the repebody with 140 μg of NHS-FITC (Thermo Scientific) in 500 μL of PBS, followed by incubation at room temperature for 2 h. Nonreacted NHS-
FITC was removed using PD-10 desalting columns (GE Healthcare). Dye-labeled repebodies were enriched using amicon ultra 10 K 0.5 mL centrifugal filters (Millipore). For labeling with horseradish peroxidase (HRP), the repebodies were incubated with HRP (EZ-link Plus Activated Peroxidase, Thermo Scientific) at a molar ratio of 2 in 500 μL of PBS (pH 7.2) at room temperature for 1 h. Immediately, 10 μL of sodium cyanoborohydride was added to increase the reduction of the Schiff base. Free HRP and repebodies were removed using amicon ultra 50 K centrifugal filters (Millipore), and the HRP-repebody was confirmed through SDS-PAGE. For multiplex imaging, each repebody specific for human, mouse, and rabbit IgG was labeled with QDS25, QDS65, and QD655 (Invitrogen), respectively, by incubating 50 pmol of QDs with 2 nmol of repebody in the presence of 200 nmol of EDC in a 10 mM HEPES (N-2-hydroxyethylpipерazine-N-2-ethanesulfonic acid) buffer (pH 7.2) at room temperature for 2 h. The QD-labeled repebodies were purified using a 100 K ultra amicon centrifuge filter.

**Cross-Reactivity Test.** For the cross-reactivity test, 30 μg/mL of IgG from different species (i.e., human, mouse, and rabbit) was coated onto a 96-well black plate (Nunc) at 4 °C overnight. Following a blocking with blocking buffer at room temperature for 1 h, FITC-labeled repebody or FITC-labeled anti-IgG antibody (Sigma-Aldrich nos. F9512, F0257, and F9887) against respective IgGs at 10 μg/mL diluted in blocking buffer was added to each well and incubated at room temperature for 1 h. After washing three times with PBST, the fluorescence intensity was measured using an Infinite N200 plate reader (Tecan) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

**ELISA Using HRP-Labeled Repebody.** To test the utility of HRP-labeled repebody for ELISA, a 0.5 μg/mL primary antibody was added to a 96-well Maxisorp plate (SPL Life Science) and incubated at 4 °C overnight. After blocking with 1% BSA in PBST at room temperature for 1 h, 100 μL of a serially diluted HRP-repebody solution was added followed by incubation at room temperature for 1 h. After washing three times with PBST, 100 μL of TMB (Sigma-Aldrich) was added to each well to generate a signal for 10 min and treated with 100 μL of 1 N H₂SO₄. The absorbance was measured using an Infinite N200 plate reader (Tecan) at 450 nm.

To determine the detection limits of the HRP-repebody against the primary antibody, a serially diluted solution of IgG with a different origin species was added to a 96-well Maxisorp plate (SPL Life Science) and incubated at 4 °C overnight. After blocking with 1% BSA in PBST at room temperature for 1 h, 100 μL of a 0.2 μg/mL HRP-repebody and HRP-conjugated secondary antibody (Sigma-Aldrich no. A0170, Abcam no. ab6789, and Biorad no. 172-1019) solution was added, followed by incubation at room temperature for 1 h. After washing three times with PBST, 100 μL of TMB (Sigma-Aldrich) was added to each well to generate a signal for 10 min and treated with 100 μL of 1 N H₂SO₄. The absorbance was measured using an Infinite N200 plate reader (Tecan) at 450 nm. The limit of detection (LOD) was calculated using the method based on the standard deviation of the response curve (Sy) and slope of the calibration curve (S) at levels approximating LOD according to the formula: LOD = 3 × (Sy/S). The standard deviation of the response was determined from the standard deviation of y-intercepts of the regression lines.

For an analysis of analytes including shiga toxin type 2 (stxB) and TNF-α through ELISA using an HRP-repebody, serially diluted analytes were coated onto a 96-well Maxisorp plate (SPL Life Science) at 4 °C overnight. After a blocking with 1% BSA in PBST at room temperature for 1 h, 0.2 μg/mL of anti-stxB mouse IgG (SantaCruz, no. 2121-70B) or anti-TNF-α antibody (etanercept) (Amgen, no. F34668) which fuses the human TNF receptor to an Fc portion of a human IgG, was added and incubated for 1 h, followed by washing three times using PBST and the addition of 0.2 μg/mL of the HRP-repebody. After incubation for 1 h at room temperature, the reaction mixture was washed three times with PBST, and 100 μL of TMB (Sigma-Aldrich) was added to each well to generate a signal for 10 min and treated with 100 μL of 1 N H₂SO₄. The absorbance was measured as described above.

**Western Blot Analysis Using HRP-Labeled Repebody.** To examine the utility of HRP-labeled repebody in Western blot analysis, 10 μg of cell lysates were separated on a 0.1% (w/v) sodium dodecyl sulfate-10% (w/v) polyacrylamide gel (10% SDS-PAGE) in SDS running buffer (30 mM Tris, 220 mM glycine, 0.1% SDS) for 1 h 30 min at 100 V, followed by transferring the proteins from the gel to a nitrocellulose membrane (Biorad, no. 162-0167) in transfer buffer (25 mM Tris, 190 mM Glycine, 20% methanol) for 2 h at 80 V. The membrane was blocked with blocking buffer at room temperature for 1 h, followed by incubation of each primary antibody (anti-Her2 human IgG (Trastuzumab) (5 μg/mL) (Roche, no. H4535H02), anti-β-actin mouse IgG (1 μg/mL) (SantaCruz, no. sc-47778), and antiphospho-ERK1/2 rabbit IgG (0.2 μg/mL) (Cell Signaling, no. 9101)) diluted in blocking buffer at room temperature for 1 h. The membranes were washed three times with PBST for 15 min and incubated with the HRP-repebody (2 μg/mL or 0.5 μg/mL diluted in blocking buffer) at room temperature for 1 h. After washing three times with PBST, the membranes were treated with an enhanced chemiluminescence (ECL) solution (Millipore) for 30 s, and signals were detected for 30 s using CCD (Biorad, ChemIDoc XRS+ system).

**Multiplex Imaging of Cells Using QD-Labeled Repebody.** For multiplex imaging with QD-labeled repebody, human epithelial carcinoma (A431), human breast cancer (MCF-7), and human breast cancer (SK-BR-3) cells were cultured in 8-well chamber slides (SPL) for 48 h, followed by washing with Dulbecco’s phosphate-buffered saline (DPBS). A mixture of primary antibodies (2 μg of anti-EGFR human antibody (Cetuximab) (Merck serono, no. ETS090812B), anti-EpCAM mouse IgG (Cell Signaling, no. 2929S), and anti-Her2 rabbit IgG (SantaCruz, no. sc-284)) in 200 μL of Hyclone RPMI-1640 media (GE Healthcare) was added to the cells and incubated at 4 °C for 1 h. Following washing twice with DPBS, a mixture of the respective repebodies labeled with different color-emitting QDs (20 nM of QDS25-rF4, QDS65-rA6, and QD655-rE2) in 200 μL of Hyclone RPMI-1640 media (GE Healthcare) was added and incubated at 4 °C for 1 h. The cells were washed three times with DPBS and fixed with 4% paraformaldehyde and DAPI staining. The fluorescent images were acquired using a confocal laser microscope (LSM NLO 710, Carl Zeiss, Germany).

**RESULTS AND DISCUSSION**

**Selection of Specific Repebodies for Mouse and Rabbit IgG.** To develop a repebody that specifically binds to mouse and rabbit IgGs, which are widely used in immunoassays, we constructed a repebody library by randomizing either variable sites on LRR modules or the C-terminal region to...
select repebodies with minimal cross-reactivity for mouse and rabbit IgGs. For selection of a repebody specific for mouse IgG, a library was constructed at six variable sites on the C-terminal loop (residues from 240 to 245) followed by a phage display selection. Three clones (rE9, rF1, and rH12) were first selected against mouse IgG, and their binding affinities for mouse IgG were determined through ITC, and their binding affinities ranged from 29 nM to 793 nM (Figure S1A). Interestingly, a preliminary test on the three initially selected clones using ELISA revealed that they have negligible cross-reactivity toward rabbit IgG. Of them, we chose rH12 to further increase the binding affinity for mouse IgG because it has the lowest affinity against human IgG. For affinity maturation, we randomized six additional variable sites at two nearby modules (LRR1 and LRRV1) of rH12 (Figure 1A) and selected three clones showing a higher affinity for mouse IgG. Among them, rA6 was shown to have the highest binding affinity of 16 nM for mouse IgG (Figure 1B, Figure S2). Using the same approach, we selected a repebody specifically binding to rabbit IgG. Briefly, a library was constructed at six variable sites on LRRV2 and LRRV3 modules followed by a phage display, and rE7 was selected to have a binding affinity of around 610 nM for rabbit IgG. We further randomized six variable sites on LRRV5 and LRRVE of rE7 for affinity maturation through a modular evolution approach (Figure 1A) and finally selected rE2 showing a binding affinity of 74 nM against rabbit IgG (Figure 1B, Figure S2). We previously developed a repebody, rF4, against human IgG, which showed a binding affinity of about 190 nM, and used it along with rA6 and rE2 in the present study.

We tested the cross-reactivity of the repebodies, namely, rA6, rE2, and rF4, against whole type IgGs with a different origin (i.e., mouse, rabbit, and human). Each whole-type of IgG is composed of whole subclasses such as IgG1, IgG2, IgG3, and IgG4. To use the repebodies for immunoassays and imaging in a multiplex manner, repebodies with high specificity for respective IgG are desirable, while showing a negligible cross-reactivity against other IgGs. We labeled each repebody with NHS-FITC through a primary amine coupling reaction, and checked their cross-reactivity through direct immunofluorescence assay. The average dye-to-repebody ratio was estimated to range from 1 to 3. As shown in Figure 2, rF4 exhibited the highest signal for human IgG and displayed a low cross-reactivity against mouse and rabbit IgGs. Similarly, rA6 and rE2 gave rise to the highest fluorescence signals specifically for the mouse and rabbit IgGs, respectively. Overall, the repebodies were shown to have cross-reactivity ranging from 7% to 16%. For comparison, we tested cross-reactivity of anti-IgG antibodies toward whole-type IgGs with a different origin using same procedure as repebodies (Figure S3). Antihuman IgG antibody and antirabbit IgG antibody showed cross-reactivities between 2% and 7%, respectively, whereas antimouse IgG antibody exhibited rather high cross-reactivity ranging from 8% to 16%.

Figure 1. Selection of repebodies against mouse and rabbit IgGs through a phage display. (A) Randomized sites for a library construction and affinity maturation for selecting a repebody for mouse and rabbit IgGs are shown. For selecting a repebody against mouse IgG, six variable sites on the C-terminal loop were randomized, and additional six variable sites on LRR1 and LRRV1 were randomized for affinity maturation. For a repebody against rabbit IgG, six variable sites on LRRV2 and LRRV3 modules were randomized, followed by randomization of additional six variable sites on LRRV5 and LRRVE modules for affinity maturation. (B) Sequences and binding affinities of selected repebodies against the respective IgGs.

Figure 2. Specificity of selected repebodies for respective IgGs: 30 μg/mL of each IgG was coated onto 96-well Maxisorp plate, followed by addition of 10 μg/mL of an FITC-labeled repebody, and the fluorescence signals were measured after three times PBST washing. Each point was tested in triplicate.
On the basis of the results, it is likely that the repebodies can be effectively used in various types of immunoassays, even though their cross-reactivities were comparable to or relatively higher than anti-IgG antibodies.

**Direct-ELISA Using HRP-Labeled Repebody.** To assess the utility of the repebodies for ELISA, we conjugated the HRP to the respective repebodies (i.e., rA6, rE2, and rF4) through a primary amine coupling reaction, yielding HRP-rA6, HRP-rE2, and HRP-rF4, respectively (Figure S4). Lysine residues in a convex region of a repebody were used for coupling with aldehyde groups of HRP through a Schiff base formation (Figure S5). Such conjugation seems to have a negligible effect on the binding affinity of HRP-conjugated repebody for IgG. After conjugation reaction, free repebody and HRP were removed using Ultra-amicon filter (50 kDa), and HRP-conjugated repebodies were analyzed by SDS-PAGE (Figure S4). Considering molecular mass of a repebody and HRP, the blur band of 70 kDa corresponds to the repebody-HRP conjugates with a 1:1 ratio. The bands above 70 kDa indicate conjugation of multiple HRP molecules to a repebody. In a conventional ELISA, an HRP-conjugated secondary antibody (HRP-antibody) is used within the range of 10 to 1 000 ng/mL. On the basis of this, 0.5 μg/mL of each IgG was coated onto a 96-well Maxisorp plate, and the concentration of HRP-conjugated repebody (HRP-repebody) varied from 1 to 2 000 ng/mL. As shown in Figure 3A, the signals increased with an increasing concentration of each HRP-repebody, showing a typical “S-shaped” curve with pseudolinear region in the middle, approaching a plateau at 2000 ng/mL of HRP-repebody. No signals were observed from BSA which was used as a negative control. HRP-rA6 specific for mouse IgG showed a relatively lower signal compared to the other HRP-repebodies although rA6 was shown to have the highest binding affinity for mouse IgG. To increase the binding affinity of a repebody for mouse IgG, we randomized the N-terminal region rather than LRRV4 or LRRVe which had been used for increasing the binding affinity of a repebody against rabbit IgG. Four lysine residues used for coupling with aldehyde groups of HRP are located at the N-terminal helix near randomized region of the repebody. It is thus likely that HRP conjugated to rA6 caused a steric hindrance to the binding of mouse IgG, resulting in a relatively low signal compared to the other HRP-repebodies in ELISA. However, the signal at 0.2 μg/mL of HRP-rA6 was sufficient for a detection of the analytes through ELISA. In this study, the level of the HRP-repebody was fixed at 0.2 μg/mL in the immunoassays. To determine the detection limit of the

**Figure 3.** ELISA using HRP-repebodies. (A) Dependency of the HRP signals on the HRP-repebody concentration: 0.5 μg/mL of each IgG was coated onto a 96-well Maxisorp plate, and different concentrations of an HRP-repebody were added. (B) Assay of human, mouse, and rabbit IgGs using respective HRP-repebodies. Serially diluted each IgG was coated onto a 96-well Maxisorp plate, and 0.2 μg/mL of the HRP-repebody was added for the assay. (C) ELISA of TNF-α and shiga toxin type 2 (stxB) using an HRP-repebody. Serial diluted analytes were coated onto a 96-well Maxisorp plate, followed by the addition of 0.2 μg/mL of antihuman TNF-α antibody (etanercept) or anti-stxB mouse IgG. After washing and treatment with 0.2 μg/mL of HRP-rF4 or HRP-rA6, the TMB reaction was carried out for 10 min. Each point was tested in triplicate.
Figure 4. Western blot analysis using HRP-repebodies. (A) Analysis of endogenous Her2 (180 kDa) in MCF-7 and SK-Br-3 cells: 10 μg of cell lysates were loaded into SDS-PAGE, followed by the transfer of proteins onto nitrocellulose membrane and the addition of 0.2 μg/mL of anti-Her2 human antibody. Subsequently, 2 μg/mL of HRP-rF4 was added and incubated for signal generation for 1 h. (B) Analysis of β-actin protein (44 kDa): 10 μg of various cancer cell lysates was loaded into SDS-PAGE, followed by the transfer of proteins onto nitrocellulose membrane and the addition of 0.2 μg/mL anti-β-actin mouse IgG. After incubation for 1 h, 0.5 μg/mL of HRP-rA6 was added and incubated for signal generation for 1 h. (C) Analysis of phosphorylated ERK1/2 (44 kDa and 42 kDa): 1 μg/mL of antiphospho-ERK1/2 rabbit IgG was added and incubated for signal generation for 1 h. Subsequently, 2 μg/mL HRP-rE2 was added and incubated for signal generation for 1 h. Phosphorylated ERK1/2, namely, pERK1 and pERK2, with a different molecular size is indicated by an arrow.

HRP-repebody for IgG, we coated different concentrations of IgG onto a 96-well Maxisorp plate and measured the signals by adding 0.2 μg/mL of respective HRP-repebody (Figure 3B). As a result, the use of 0.2 μg/mL repebodies gave rise to a comparable detection limit to HRP-antibodies. On the basis of the result, it is likely that respective HRP-repebodies can be used in immunoassays.

To assess the utility of the HRP-repebodies in immunoassays, we assayed other analytes, including TNF-α and shiga toxin type 2 (stxB), using HRP-rF4 and HRP-rA6, respectively. TNF-α is involved in inflammation, and a shiga toxin secreted by E. coli strains is known to cause a variety of clinical syndromes including diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS).26 Both TNF-α and stxB are commonly analyzed through conventional ELISA.18 Unlike conventional sandwich-ELISA using capture antibody, we simply tested direct-ELISA using HRP-repebodies to detect TNF-α and stxB. Serially diluted TNF-α or stxB was coated onto a 96-well Maxisorp plate, and the respective primary antibodies, namely, anti-TNF-α antibody and anti-stxB mouse IgG were added. As shown in Figure 3C, HRP-rF4 generated a signal for TNF-α as low as 9.9 ng/mL and 7.8 ng/mL for TNF-α and stxB, respectively (Figure S6). These results demonstrate that HRP-repebodies can be used as alternatives to conventional secondary antibodies for immunoassays for a variety of analytes.

Western Blot Analysis Using HRP-Repebody. We tested a HRP-repebody for use in a Western blot analysis, and chose Her2, β-actin, and phospho-ERK1/2 as the target proteins. HRP-rF4, HRP-rA6, and HRP-rE2 were used as alternatives to the secondary antibodies against anti-Her2 human IgG, anti-β-actin mouse IgG, and antiphospho-ERK1/2 rabbit IgG, respectively, in a variety of cell lines: SK-Br-3 (human breast cancer cells) overexpressing Her2, MCF-7 with a low Her2 expression, A431 (human squamous carcinoma), HeLa (human nevoid cervix epithelioid carcinoma), Panc-1 (human Cauca- lain pancreas), HT-29 (human colorectal adenocarcinoma), and U2OS (human Bone Osteosarcoma) cell lines. For the Western blot analysis, each cell was lysed and subjected to SDS-PAGE, followed by the addition of primary antibodies and subsequent treatment with respective HRP-repebodies against the primary antibodies (Figure 4). A Western blot analysis of endogenous Her2 receptors using HRP-rF4 showed a clear band at the expected position (Figure 4A), and the use of HRP-labeled anti-human IgG antibody also resulted in an expected band (Figure S7A). In addition, the signals from HRP-rF4 were shown to be closely related to the expression levels of endogenous Her2 receptors in the cells: a strong signal from SK-Br3 expressing a high level of Her2, and a negligible signal from MCF-7 which was known to express no Her2. An analysis of β-actin in various cancer cell lines using HRP-rA6 also resulted in distinct bands at the corresponding position (Figure 4B), exhibiting a comparable or better performance compared to the HRP-labeled antimouse IgG antibody (Figure S7B). We also applied HRP-rE2 to phosphorylated ERK 1/2 (Extracellular signal-regulated kinase 1/2) in different cell lines. ERK1/2 is known to control a broad range of cellular activities and physiological processes.29 The primary antibody against the phosphorylated ERK used was revealed to recognize phosphorylated ERK1/2 with different molecular masses of 44 and 42 kDa, respectively.30 As shown in Figure 4C, two bands corresponding to the respective phosphorylated ERK1/2 (pERK1 and pERK2) were clearly observed using HRP-rE2. On the other hand, the use of HRP-labeled antirabbit IgG antibody gave rise to weak pERK1/2 bands as well as other nonspecific bands (Figure S5C). This result seems to come from high accessibility or low steric hindrance of HRP-repebody with primary antibodies compared to HRP-antibody. The size of a repebody (∼30 kDa) is approximately one-fifth of antibody, and a repebody with a smaller size seems to have high accessibility or low steric hindrance to the primary antibody, giving rise to two bands corresponding to pERK1 and 2, respectively. On the basis of the results, it is plausible that the repebodies specific for IgG with a different origin can be used for a Western blot analysis of a variety of proteins in diverse cell lines, such as cell-surface receptors, intracellular proteins, and phosphorylated proteins, with high specificity.

Multiplex Imaging Using QD-Labeled Repebody. To assess the utility of an anti-IgG repebody for imaging of cell-surface proteins in a multiplex manner, three repebodies (rF4, rA6, and rE2) were conjugated with different color-emitting quantum dots, namely, QD525, QD565, and QD655, yielding QD525-rF4, QD565-rA6, and QD655-rE2, respectively. Because quantum dots have a broad excitation band and a narrow emission band, the use of different color-emitting quantum dots enables the detection of targets in a multiplex manner. The conjugation of each quantum dot to the respective repebody was confirmed through a gel shift assay (Figure S8).
We tested an epidermal growth factor receptor (EGFR), an epithelial cell adhesion molecule (EpCAM) and Her2 as the targets for multiplex imaging using rF4, rA6, and rE2, respectively. Multiplex detection of these target proteins on the same cells is of great significance for the discrimination of cancer cells. Cells expressing EGFR, EpCAM, and Her2 were treated with a mixture of the respective primary antibodies against EGFR, EpCAM, and Her2, followed by the addition of a mixture of three different QD-repebodies. As shown in Figure 5, cell-surface proteins, namely, EGFR, EpCAM, and Her2, were simultaneously visualized in SK-Br-3 cells expressing three proteins on the cell surface. The fluorescent signal from QD525 was relatively low, and this seems to be due to lower expression level of EGFR than Her2 in SK-Br-3 cells.31 To further test the specificity of repebodies in a multiplex imaging, we used other cell lines, such as HCC827 and HepG2. HCC827 cells are known to express EGFR and EpCAM but not Her2,32 whereas HepG2 cells do not express EGFR, EpCAM, and Her2.33 As a result, fluorescent signals from EGFR and EpCAM were observed only in the HCC827 cells, and no signals were detected from the HepG2 cells. It means that repebodies have a high specificity for each IgGs from different origins, respectively. In other words, repebodies do not have cross-reactivity against other species’ IgGs except for partner IgG. This result demonstrates that the developed repebodies can be effectively used for the imaging of cell-surface receptors and protein in a multiplex manner.

CONCLUSION

We demonstrated that the repebodies specific for IgG from different species (i.e., mouse, rabbit, and human) can be used in immunoassays and multiplex imaging. The utility and potential of the repebodies as alternatives to secondary antibodies were demonstrated by their analytical performance for various immunoassay formats and multiplex imaging. As for ELISA, a HRP-repebody led to a sensitive analysis of various targets, showing comparable sensitivity to conventional secondary antibodies. Moreover, the use of an HRP-repebody for a Western blot analysis enabled the sensitive and specific detection of target proteins with negligible nonspecific bands. Repebodies labeled with different color-emitting quantum dots showed distinct and clear images of receptors and protein on the cell surface in a multiplex manner. Recently, the experimental data using antibodies were revealed to lack reproducibility, which implies the need for more reliable primary and secondary antibodies.34−36 In this regard, the repebody with high specificity for IgG from different species can find wide applications as reliable alternatives to conventional secondary antibodies. Furthermore, target-specific repebodies can be easily developed using the same approach as alternatives to primary antibodies. Combination of the primary and secondary repebodies is thus anticipated to provide reliable results in immunoassays and multiplex imaging.

ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b03851.

Binding affinities data, specificity of FITC-labeled antibody, SDS-PAGE data, structure of repebody, Western blot analysis, and analysis of QD-conjugated repebodies by TB agarose gel shift assay (PDF)

AUTHOR INFORMATION

Corresponding Author
*E-mail: hskim76@kaist.ac.kr. Phone: +82-42-350-2616. Fax: +82-42-350-2610.

Author Contributions
§S.J. and W.H. equally contributed to this work.

Notes
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