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Simply Mixing Poly Protein G with Detection Antibodies Enhances the Detection Limit and Sensitivity of Immunoassays

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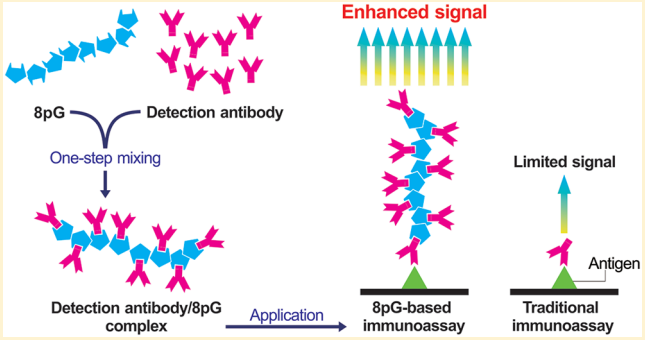
Supporting Information

ABSTRACT: An insufficient amount of detection antibodies bound to their antigens usually limits the sensitivity of immunoassays. Here, we describe a simple method to improve the detection limit and sensitivity of various immunoassays by mixing detection antibodies with a soluble poly protein G (named 8pG). 8pG was developed by fusing eight repeated fragment crystallizable (Fc) binding domains of streptococcal protein G to a linear polymer. Simply mixing detection antibodies with 8pG to form an antibody/8pG complex largely increased the accumulation of detection antibody to target molecules, which dramatically enhanced the sensitivity in direct ELISA, sandwich ELISA, Western blot, and flow cytometry systems, separately. The detection limit of Western blot for low-abundance PEGylated interferon (Pegasys) and recombinant human CTLA4 (rhCTLA4) improved by at least 13-fold and 31-fold, respectively, upon mixing detection antibodies with 8pG. Moreover, the nanoscale size of the antibody/8pG complex did not influence the granularity and dimension of target cells in the flow cytometry system. Collectively, we provide a quick and easy-to-operate method to make various immunoassays to sensitively detect low-abundance target molecules by just mixing their detection antibodies with 8pG.



With the advantages of versatility, specificity, and easy operation, immunoassays including enzyme-linked immunosorbent assay (ELISA), flow cytometry, and Western blot have been commonly used to detect the presence or to determine the concentration of analytes ranging from small molecules to macromolecules in complex biological specimens for medical research or clinical diagnosis.[1−7] Although the technical models of these immunoassays are different, the common principle behind these assays is based on the specific recognition of target molecules[8] as well as antigens[9−12] by using detection antibodies. In most cases, the antigens immobilized on solid-phase materials (e.g., 96-well plates and nitrocellulose membrane) or expressed on the cell surface are often in low abundance. Rare antigens allow only a limited accumulation of detection antibodies, leading to an insufficient signal for antigen detection in immunoassays.[12,13] Reasonably, if we can increase the amount of detection antibodies that accumulate at antigens, the detection limit and sensitivity of each immunoassay will be greatly improved.

Various kinds of antibody-coated nanoparticles or polymers have been developed to improve the sensitivity of immunoassays.[14−19] A large amount of detection antibodies can

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accumulate at one antigen by coating nanoparticles with a high density of antibodies, which effectively increases the detection signal in immunoassays.[20−24] However, the preparation of these antibody-coated nanoparticles is complicated and lacks consistency.[25−27] Chemical linkage of abundant amine groups in antibodies to cyanogen bromide (CNBr)-activated or *N*-hydroxysuccinimide (NHS)-activated nanoparticles usually causes heterogeneous orientations of the antibodies on the nanoparticles, which impair the desired antibody−antigen binding.[28,29] Although an affinity-binding nanoparticle coated with recombinant protein G can specifically trap detection antibodies on their surface with a consistent orientation,[30,31] it is cumbersome and involves a costly manufacturing process that includes the production of recombinant protein G, the chemical linkage of protein G to nanoparticles, and the purification of the protein G-coated nanoparticles.[17,32] In previous works, we developed a strain of *E. coli* bacteria that expresses surface poly protein G as antibody-trapping microparticles to augment the signal of immunoassays (ELISA and Western blot systems) by increasing the accumulation of detection antibodies to target molecules. The antibody-trapping microparticles were simply and rapidly generated by a one-step mixing of poly protein G-expressing bacteria with a detection antibody, and the mixing did not impair the antibody function. However, the poly protein G-expressing bacteria were not suitable for use in flow cytometry systems because the microscale size of bacteria alters the granularity and size of target cells, resulting in signal deviation. Therefore, developing a signal enhancer with extremely small-scale size for detection antibodies would have a wider applicability and flexibility in various immunoassay systems.

In this study, we created a soluble poly protein G (named 8pG) with high-selectivity and high-affinity binding capacity to the Fc region of IgG-type immunoglobulins for enhancing the detection limit and sensitivity of various immunoassays. 8pG was developed by fusing eight C2 domains of streptococcal protein G to a tandemly repeated polymer, which allows each 8pG molecule to trap up to eight detection antibodies on its scaffold. One-step mixing of 8pG with detection antibody can immediately generate an antibody/8pG complex, which dramatically increases the interaction and accumulation of detection antibody to target antigen (Figure 1). The expression and purity of 8pG were analyzed by Western blot and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), respectively. The antibody-trapping specificity of 8pG was assessed by adding horseradish peroxidase (HRP)-conjugated antibody to 8pG-coated or BSA-coated microplates. Furthermore, we mixed 8pG with various detection antibodies to demonstrate the enhancement of detection limit and sensitivity in different immunoassays, including direct ELISA, sandwich ELISA, Western blot, and flow cytometry.

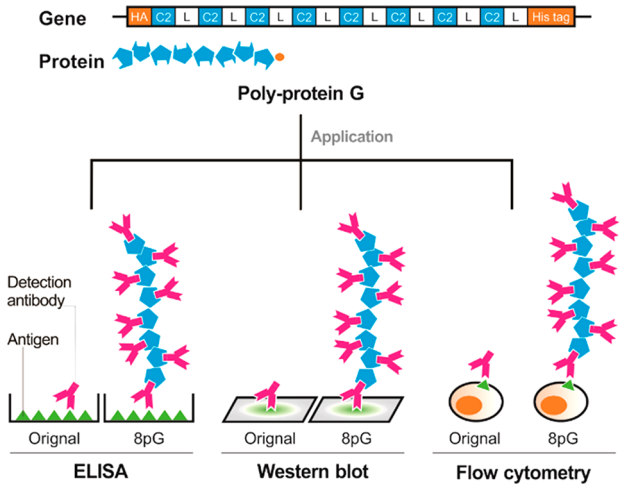

Figure 1. Schematic illustration of the eight tandemly repeated protein G C2 domain polymer (8pG). The 8pG gene includes, from N-terminus to C-terminus, an HA tag, a fragment consisting of repeated protein G C2 domains (C2) and $(GGGSG)_3$ peptide linkers (L), and a His tag. Simply mixing the 8pG with detection antibodies to generate a detection antibody/8pG complex can increase the accumulation of detection antibodies to the target molecule (green color), thus enhancing the detection signal of various immunoassays.

EXPERIMENTAL SECTION

Reagents and Cells. Restriction enzymes ClaI, *Hin*dIII, SalI, and SfiI were purchased from New England Biolabs (Ipswich, MA). ExpiFectamine 293 Transfection Kit, Expi293 expression medium, phosphate-buffered saline (PBS), Opti-MEM Reduced Serum Media, penicillin−streptomycin, and enhanced chemiluminescence (ECL) substrate for Western blot were purchased from Thermo-Fisher Scientific (San Jose, CA). PBS containing Tween-20 (PBST) was prepared by dissolving 0.05% (v/v) Tween-20 in PBS. Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin (BSA), bovine calf serum (BCS), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and polyethylene glycol with 5000 Da (PEG_{5K}) were purchased from Sigma-Aldrich (St. Louis, MO). Biotinylated mouse anti-PEG IgG monoclonal antibodies (termed 3.3-biotin) and mouse anti-PEG IgM monoclonal antibodies (termed AGP4) were offered by Dr. Steve R. Roffler (Academia Sinica, Taipei, Taiwan).[33,34] Monoclonal mouse anti-PDL1 antibodies and monoclonal mouse anti-CTLA4 antibodies were purchased from Abnova (Taipei, Taiwan). Monoclonal mouse anti-His tag antibodies were purchased from AbD Serotec (Kidlington, Oxford, UK).

Pegasys was purchased from Roche (Branchburg, NJ). Pierce BCA protein assay kit and quantum dots were purchased from Thermo-Fisher Scientific (San Jose, CA). Lipo-Dox was purchased from TTY Pharmaceutical Co. (Taipei, Taiwan). Recombinant human CTLA4 (rhCTLA4) was produced as previously described.[35] FITC-conjugated goat antimouse IgG Fcγ antibody, HRP-conjugated donkey antirabbit IgG antibody, HRP-conjugated goat antimouse IgG Fc antibody, and HRP−streptavidin were purchased from Jackson Immuno Research Laboratories (West Grove, PA). HRP-conjugated rabbit antigoat IgG antibody was purchased from Sigma-Aldrich.

HT-29 human colon cancer cells and 3T3 mouse fibroblasts were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM medium containing 10% BCS and 100 units/mL of penicillin and streptomycin at 37 °C with 5% CO_2. Expi293 cells were purchased from Thermo-Fisher Scientific and cultured in Expi293 Expression Medium at 37 °C with 8% CO_2 on a rotary shaker at 120 rpm.

Plasmid Construction. The 8pG gene was synthesized as previously described.[8,35] The eight tandemly repeats of protein G-C2 domains were subcloned with SfiI and SalI into a pLNCX secretion vector (Clontech, Mountain View, CA) containing a six-histidine (His) epitope to form the pLNCX-8pG-His plasmid.

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8pG Production and Purification. To produce 8pG, Expi293 cells were grown to a density of 7.5×10^7 cells/ml in 25.5 mL of Expi293 expression medium. pLNCX-8pG-His (30 μg) was transfected into Expi293 cells with ExpiFectamine. 8pG was harvested 5 days after the transfection, followed by centrifugation at 4000*g* for 30 min and filtration with a 0.2-μm filter flask (Millipore). Pure 8pG was obtained by using a HiTrap column (GE HealthCare) and elution with an buffer containing 500 mM imidazole. Buffer dialysis against PBS (Sigma-Aldrich) was performed to remove imidazole and unwanted salt. The concentrate of pure 8pG was measured by Pierce BCA protein assay kit (Thermo-Fisher Scientific). The purity of 8pG was analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and Coomassie blue staining.

Immunoblot Analysis of 8pG. 8pG was electrophoresed in 10% reducing SDS-PAGE and subsequently transferred onto a nitrocellulose (NC) membrane for Western blot. The membrane was blocked in 5% (w/v) milk (dissolved in PBST) and then incubated with anti-His antibody (1 μg/mL), followed by HRP-conjugated goat antimouse IgG Fc antibodies (1 μg/mL). The blotting was visualized with ECL substrate.

Analysis of Antibody-Trapping Ability of 8pG by ELISA. 8pG or BSA (500 ng/well) was coated in Maxisorp 96-well microplates (Nalge Nunc International, Rochester, NY) with coating buffer (0.1 M $NaHCO_3$, pH = 9) for 2 h at 37 °C. The microplates were blocked with 3% (w/v) BSA diluted in PBS overnight at 4 °C. The HRP-conjugated antibodies (1, 0.3, 0.1, 0.03, and 0.01 μg/mL) were added to the microplates and incubated at room temperature for 1 h. After wells were washed with PBST, HRP−streptavidin and ABTS were added and incubated sequentially in the microplates. Absorbance at 405 nm was measured by a microplate reader.

8pG-Based ELISA. For anti-PEG ELISA, serially diluted PEG_{5K}-NH_2 (0.69, 2, 6.2, 18.5, 55.5, 166, and 500 ng/well) was coated in Maxisorp 96-well microplates with the coating buffer for 2 h at 37 °C, and then the microplates were blocked with 3% (w/v) BSA in PBS overnight at 4 °C. Biotinylated anti-PEG antibodies (6, 2, 0.66, 0.22, and 0.08 μg/mL of 3.3-biotin) were mixed individually at a 1:1 volume ratio with 8pG at room temperature for 2 h (thus, the final concentrations of 3.3-biotin were 3, 1, 0.33, 0.11, and 0.04 μg/mL). The 8pG/3.3-biotin mixtures were added to microplates and incubated at room temperature for 1 h. After wells were washed with PBS, HRP−streptavidin and ABTS were added and incubated sequentially in the microplates to detect PEG_{5K}-NH_2. Absorbance at 405 nm was measured by a microplate reader.

For anti-rhCTLA4 ELISA, serially diluted rhCTLA4 (18.5, 55.5, 166, and 500 ng/well) was coated in Maxisorp 96-well microplates with the coating buffer for 2 h at 37 °C, and then the microplates were blocked with 3% (w/v) BSA in PBS overnight at 4 °C. The anti-CTLA4 antibodies (Abnova, Taipei, Taiwan) (6, 2, 0.66, 0.22, and 0.074 μg/mL) were mixed individually at a 1:1 volume ratio with 8pG at room temperature for 2 h (thus, the final concentrations of anti-CTLA4 antibody were 3, 1, 0.33, 0.11, and 0.037 μg/mL). The 8pG/anti-CTLA4 antibody mixtures were added to microplates and incubated at room temperature for 1 h. After wells were washed with PBS, HRP-conjugated goat antimouse IgG Fc (1 μg/mL), and ABTS were added and incubated sequentially in the microplates to detect rhCTLA4. Absorbance at 405 nm was measured by a microplate reader.

8pG-Based Sandwich ELISA. An anti-PEG sandwich ELISA kit using a capture/detection pairing of AGP4/3.3-biotin was used to measure PEGylated human IFN-α (Pegasys), PEGylated liposomal doxorubicin (Lipo-Dox), and PEGylated quantum dots (Q-dots). The capture antibody AGP4 (1 μg/mL) diluted in the coating buffer was coated in Maxisorp 96-well microplates for 2 h at 37 °C, and then the microplates were blocked with 3% (w/v) BSA in PBS overnight at 4 °C. Pegasys, Lipo-Dox, or Q-dots was serially diluted in 0.05% (w/v) BSA in PBS and then incubated in microplates for 2 h at room temperature. The detection antibody 3.3-biotin (2 μg/mL) was mixed at a 1:1 volume ratio with 8pG at room temperature for 2 h (thus, the final concentration of 3.3-biotin was 1 μg/mL). The 8pG/3.3-biotin mixtures were added to microplates and incubated at room temperature for 1 h. After wells were washed with PBS, HRP-conjugated streptavidin and ABTS were added and incubated sequentially in the microplates to detect PEG-conjugated antigens. Absorbance at 405 nm was measured by a microplate reader.

An IFN-α ELISA development kit (Mabtech AB, Germany) with a capture/detection pairing of MT1/MT2 was used to measure human IFN-α. The capture antibody MT1 (1 μg/mL) was coated to Maxisorp 96-well microplates in the coating buffer for 2 h at 37 °C, and then the microplates were blocked with 3% (w/v) BSA in PBS overnight at 4 °C. Human IFN-α were serially diluted in 0.05% (w/v) BSA in PBS and then incubated in microplates for 2 h room temperature. The detection antibody MT2 (2 μg/mL) was mixed at a 1:1 volume ratio with 8pG at room temperature for 2 h (thus, the final concentration of MT2 was 1 μg/mL). The 8pG/MT2 mixtures were added to microplates and incubated at room temperature for 1 h. After wells were PBS, HRP-conjugated streptavidin, and ABTS were added and incubated sequentially in the microplates to detect human IFN-α. Absorbance at 405 nm was measured by a microplate reader.

8pG-Based Western Blot. For anti-PEG Western blot, Pegasys was electrophoresed in reducing 10% SDS-PAGE and subsequently transferred onto a NC membrane. The blots were blocked in 5% (w/v) milk in PBST for 2 days at 4 °C. 3.3-biotin (2 μg/mL) was mixed at a 1:1 volume ratio with 8pG at room temperature for 2 h (thus, the final concentration of 3.3-biotin was 1 μg/mL). The 8pG/3.3-biotin mixtures were added to NC membrane and incubated at room temperature for 1 h. After the membrane was with PBS, HRP−streptavidin and ECL substrate were used to detect Pegasys.

For anti-rhCTLA4 Western Blot, the rhCTLA4 was electrophoresed in reducing 10% SDS-PAGE and subsequently transferred onto a NC membrane. The membrane was blocked in 5% (w/v) milk in PBST for 2 days at 4 °C. The anti-CTLA4 antibody (2 μg/mL) was mixed 1:1 with 8pG at room temperature for 2 h (thus, the final concentration of anti-CTLA4 antibody was 1 μg/mL). The 8pG/anti-CTLA4 antibody mixtures were added to the membrane at room temperature for 1 h. After the membrane was washed, HRP-conjugated goat antimouse IgG Fc antibody (1 μg/mL; Jackson ImmunoResearch) and ECL substrate were added to detect rhCTLA4.

8pG-Based Flow Cytometry. HT-29 cells (PD-L1$^+$) were suspended at 3×10^5 cells/tube in 0.05% (w/v) BSA in PBS. The anti-PDL1 antibody (Abnova, 2 μg/mL) was mixed at a 1:1 volume ratio with 8pG at room temperature for 2 h (thus, the final concentration of anti-PDL1 antibody was 1 μg/mL).

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The 8pG/anti-PDL1 antibody mixtures were incubated with HT-29 cells at 4 °C for 1 h. Then HT-29 cells were incubated with FITC-conjugated goat anti mouse IgG Fc antibody (1 μg/mL) at 4 °C for 1 h. After PBS washing, forward scatter (FSC) signal, side scatter (SSC) signal, and FITC fluorescence of the cells were measured by a flow cytometer (BD Biosciences, San Jose, CA), and the data were analyzed with Flowing Software created by Perttu Terho.

Statistical Analysis. All experiments were repeated at least twice with representative data shown. In ELISAs, all of the absorbance were background corrected by subtracting the blank value. In Figures 3 and 4, two-way ANOVA was performed to calculate the statistical differences between the groups. Independent *t* test was performed to verify significant difference in the detection limits between the traditional ELISAs and the 8pG-based ELISAs. Results were considered statistically significant if $p \leq 0.05$.

RESULTS AND DISCUSSION

Development and Characterization of Poly Protein G (8pG). Recombinant protein G from streptococcal bacteria can specifically bind to immunoglobulins, especially IgG types, of various species which has been widely used in antibody purification technology.[36−39] Native protein G contains two distant protein binding sites including N-terminal albumin binding region and C-terminal immunoglobulin binding region.[40−44] The immunoglobulin binding region can subdivide into C1, C2, and C3 domains. Only the C2 domain can specifically bind the Fc region[45,46] while the C1 and C3 domains bind both Fab and Fc regions of immunoglobulins. In addition, most current commercial detection antibodies used in various immunoassays are produced by specific species such as mouse, rat, goat, horse, rabbit, and sheep[7,47,48] which can all be recognized by protein G C2 domain.[37,38]

Here, 8pG was designed as a polymer of eight tandemly repeated protein G C2 domains linked with a C-terminal six-His tag, which can specifically trap the Fc region of most commercial detection antibodies without interfering with their antigen binding ability for various immunoassay systems (Figure 1). Recombinant 8pG could be massively produced by Expi-293 producer cells in a yield of >100 mg/L. Western blot analysis using anti-His tag antibody showed that 8pG was expressed in an expected size of 60 kDa under reducing conditions (Figure 2A). SDS-PAGE analysis showed the high purity of 8pG after Ni-affinity chromatography purification (Figure 2B). The antibody-trapping ability of 8pG was confirmed by staining the 8pG- or BSA-coated microplates with HRP-conjugated IgG immunoglobulins of different species, including goat, mouse, donkey, and rabbit. Figure 2C shows that all of these immunoglobulins could be specifically trapped on the 8pG-coated microplate but not the BSA-coated control microplate. These results indicate that recombinant 8pG with specific antibody-trapping ability can be easily produced and purified.

Due to the specific binding of protein G C2 domain to the Fc region of mostly IgG, the 8pG-based immunoassays may not be suitable for detecting certain IgG type antigens which can compete with detection antibodies for the Fc binding sites on the 8pG scaffold.[45,49] In the same way, some blocking reagents, such as bovine serum, containing immunoglobulins also slightly impaired the detection sensitivity and increased background noise of 8pG-based immunoassays. Even so, the 8pG-based immunoassays with BCS blocking were still more

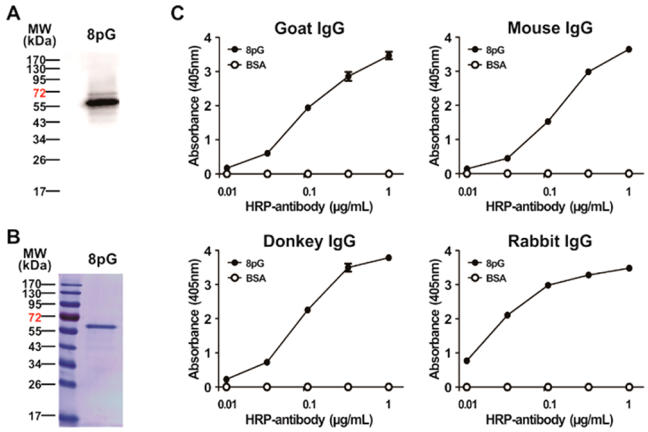

Figure 2. Characterization of 8pG. (A) The expression of 8pG was assessed by Western blot with mouse anti-His tag antibodies and HRP-conjugated goat antimouse IgG Fc antibodies. (B) Reducing SDS-PAGE showed Coomassie blue staining of purified 8pG. (C) The IgG-type immunoglobulin trapping ability was assessed by adding HRP-conjugated goat, mouse, donkey, or rabbit antibodies into 8pG- or BSA-coated microplates, respectively. Bar, SD.

sensitive than the traditional immunoassays (Figure S1). Collectively, we eventually chose pure bovine serum albumin (BSA) as the diluent and blocking reagents for 8pG-based immunoassays including ELISA, flow cytometry, and Western blot systems.

Simple Mixing of Detection Antibodies with 8pG Enhances the Detection Signal of Direct ELISA. To evaluate whether the 8pG is able to improve the detection limit and sensitivity of direct ELISA, we added 1 μg/mL of biotinylated anti-PEG antibody (termed 3.3-biotin) mixed with or without 8pG to the microplates coated with different amounts of the PEG_{5K}-NH_2 (0.69, 2, 6.2, 18.5, 55.5, 166, and 500 ng/well), followed by the sequential addition of HRP−streptavidin and ABTS substrate. Figure 3A shows that the absorbance of the 8pG group (3.3-biotin mixed with 8pG) for detecting different amounts of PEG_{5K}-NH_2 (6.2−500 ng/well) were 11.52-fold, 3.63-fold, 1.6-fold, 1.5-fold, and 1.2-fold, respectively, higher than those of 3.3-biotin-alone group. The 8pG-based groups could sensitively detect PEG_{5K}-NH_2 at concentrations as low as 0.69 ng/well, whereas the detection limit of the 3.3-biotin groups was 6.2 ng/well. On the other hand, we added the different concentrations (0.04, 0.11, 0.33, 1, and 3 μg/mL) of 3.3-biotin mixed with or without 8pG to the PEG_{5K}-NH_2-coated microplates (20 ng/well). Figure 3B shows that the absorbance values of the 8pG groups (0.33, 1, and 3 μg/mL of 3.3-biotin mixed with 8pG) for detecting PEG_{5K}-NH_2 were 7.8-fold, 2.2-fold, and 1.4-fold higher than those of 3.3-biotin-alone groups.

We also applied 8pG in another direct ELISA system for detecting soluble recombinant human CTLA4 (rhCTLA4). We added 1 μg/mL of anti-CTLA4 antibody mixed with or without 8pG to the microplates coated with different amounts of rhCTLA4 (18.5, 55.5, 166, and 500 ng/well), followed by sequential addition of the secondary antibody and ABTS substrate. Figure 3C shows that the 8pG groups and the anti-CTLA4 antibody-alone groups showed no statistical differences in detecting the 166 and 500 ng/well doses of rhCTLA4. However, at the 55.5 ng/well dose of rhCTLA4, the 8pG-based group was significantly higher (3.1-fold) than the anti-CTLA4 antibody-alone group. The 8pG-based groups could

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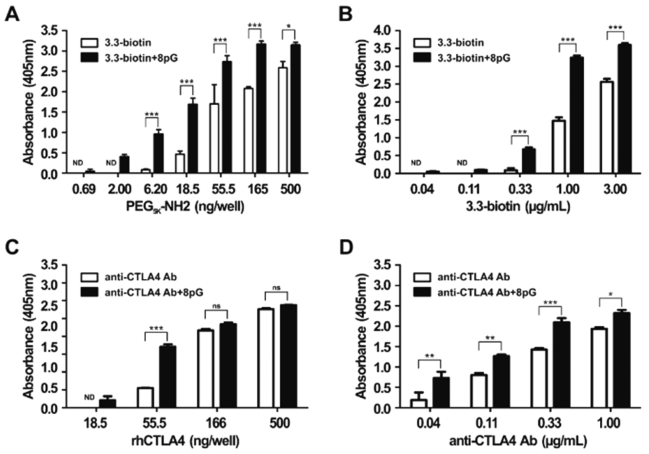

Figure 3. Direct ELISAs performed in the presence and absence of 8pG. (A) 3.3-biotin (1 μg/mL) mixed with or without 8pG was added into microplates coated with graded amounts of PEG_{5K}-NH_2 molecules. (B) Graded concentration of 3.3-biotin mixed with or without 8pG was added into microplates coated with PEG_{5K}-NH_2 molecules (20 ng/well). (C) Anti-CTLA4 antibody (1 μg/mL) with or without 8pG was added into microplates coated with graded amounts of rhCTLA4 molecules. (D) Graded concentration of anti-CTLA4 antibody mixed with or without 8pG was added into microplates coated with rhCTLA4 molecules (55.5 ng/well). Bar, SD *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$. ns, no significant difference. ND, not detected.

sensitively detect rhCTLA4 at doses as low as 18.5 ng/well. Figure 3D shows that the absorbance of serially diluted anti-CTLA4 antibody (0.037, 0.11, 0.33, and 1 μg/mL) mixed with 8pG for detecting rhCTLA4 (55.5 ng/well) was 3.9-fold, 1.6-fold, 1.5-fold, and 1.2-fold higher than the value of the anti-CTLA4 antibody-alone groups.

Interestingly, 8pG dramatically enhances the sensitivity of antibodies to detect PEG_{5K}-NH_2 or rhCTLA4 at low density but not at high density in direct ELISA tests. It is speculated that at high antigen density, if an antibody/8pG complex binds an antigen in a "lying down" position, it will mask nearby antigens and make other antibodies unable to bind to the masked antigens, resulting in limited detection sensitivity. Conversely, in the case of low antigen density, no matter whether an antibody/8pG complex is "standing upright" or "lying down", it does not interfere with the binding of other antibodies to nearby antigens (models are shown in Figure S2). This phenomenon was also found in other signal enhancers such as antibody-conjugated nanoparticles and polymers for ELISAs.[50,51] Notably, even if antibody/8pG complexes had steric hindrance on identifying high abundant antigens, the detection sensitivity of 8pG groups was still better than traditional antibody-alone groups.

Simple Mixing of Detection Antibodies with 8pG Enhances the Detection Signal of Sandwich ELISA. We further examined whether 8pG improves the detection limit and sensitivity of different commercial sandwich ELISA kits, namely an anti-PEG sandwich ELISA kit and an anti-IFN-α sandwich ELISA kit, to detect various analytes. For the anti-PEG sandwich ELISA kit, serial diluted PEGylated human IFN-α (Pegasys), PEGylated liposomal doxorubicin (Lipo-Dox), or PEGylated quantum dots (Q-dots) were added into microplates coated with an IgM type anti-PEG antibody (termed AGP4), followed by the sequential addition of 3.3-biotin mixed with or without 8pG, HRP−streptavidin, and ABTS substrate. Figure 4A shows that the absorbance values of the 8pG group to detect Pegasys (0.1, 1, 10, 100, and 1000 ng/

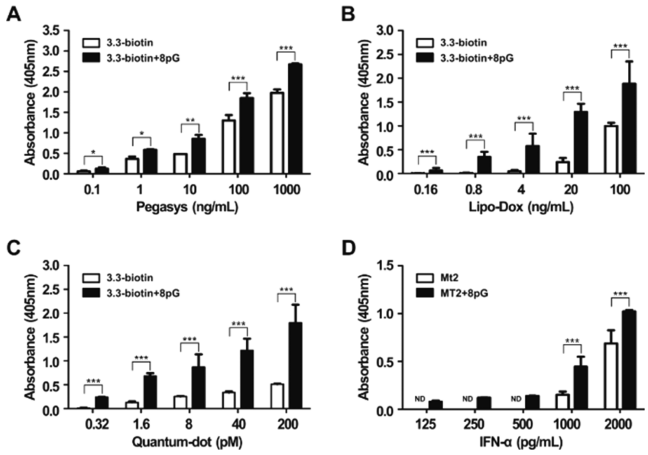

Figure 4. Sandwich ELISA tests performed in the presence and absence of 8pG. Graded concentration of (A) Pegasys, (B) Lipo-Dox, or (C) Q-dots was added into microplate coated with an IgM type anti-PEG antibody (AGP4). Captured antigens were detected by 3.3-biotin mixed with or without 8pG, and then color was developed using ABTS. Graded concentration of (D) IFN-α was added into microplate coated with MT1 anti-IFN-α capture antibody. The captured IFN-α was detected with MT2 biotinylated anti-IFN-α detection antibody mixed with or without 8pG, and then color was developed using ABTS. Bar, SD *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$. ND, not detected.

mL) were 2-fold, 1.6-fold, 1.8-fold, 1.4-fold, and 1.4-fold higher than that of the 3.3-biotin-alone group. Figure 4B shows that the absorbance values of the 8pG groups to detect Lipo-Dox (0.16, 0.8, 4, 20, and 100 ng/mL) were 19-fold, 49.8-fold, 11.6-fold, 5.3-fold, and 1.9-fold higher than that of the 3.3-biotin-alone group. Figure 4C shows that the absorbance values of the 8pG group to detect Q-dots (0.32, 1.6, 8, 40, and 200 pM) were 16.4-fold, 5.2-fold, 3.4-fold, 3.6-fold, and 3.5-fold higher than that of the 3.3-biotin-alone group. These data indicate that 8pG could significantly enhance the anti-PEG antibody-based sandwich ELISA kit for highly sensitive measurement of PEG-conjugated macromolecules containing different core materials, such as proteins, liposomes, and inorganic nanoparticles.

For the anti-IFN-α sandwich ELISA kit, defined concentrations of human IFN-α (125, 250, 500, 1000, and 2000 pg/mL) were added into microplates coated with the anti-IFN-α antibody (termed MT1), followed by the sequential addition of a biotinylated anti-IFN-α antibody (termed MT2) mixed with or without 8pG, HRP-streptavidin, and ABTS substrate. Figure 4D shows that the absorbance values of the 8pG group to detect 1000 and 2000 pg/mL of IFN-α were 2.9-fold, and 1.5-fold higher than that of the MT2-alone group. Moreover, the 8pG group showed a higher sensitivity as low as 125 pg/mL of human IFN-α, whereas the MT2-alone group failed to detect 500 pg/mL (and below) of the antigen.

There are kinds of strategies to effectively enhance the detection limit and sensitivity of the sandwich ELISA systems or sandwich-like immunoassays. In this study, the 8pG's strategy is based on increasing the amount of detection antibodies accumulating at the antigen site. Furthermore, some researchers focused on improving the detection signal of immunoassays by using the enzyme cascade amplification

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strategy for colorimetric signal enlargement[52] or using the energy-transfer conversion strategy via photoelectric materials to amplify the photoelectrochemical signal.[53−55] Another group of researchers focused on improving the amount and "displaying orientation" of capture antibodies immobilized on the ELISA microplate precoated with chromium(III)[56] or hydrophilic polyelectrolyte multilayer substrates.[57] We believe that 8pG can easily and nonconflictingly cooperate with these signal amplification strategies to further increase the efficiency and quantity of antibody binding to antigen, making the sandwich-type immunoassays ultrasensitive.

Simple Mixing of Detection Antibodies with 8pG Enhances the Detection Signal of Western Blot. Western blot is a widely used technique to identify a specific protein separated from complex biological specimens according to molecular weight. However, the detection limit and sensitivity of Western blot are often constricted by insufficient detection antibodies bound to a small amount of targeted proteins in the transferred membrane.[58] Therefore, we applied 8pG as a signal enhancer in two Western blot systems for detecting Pegasys by using 3.3-biotin antibody or detecting rhCTLA4 by using anti-CTLA4 antibody. Figure 5A shows that the signal

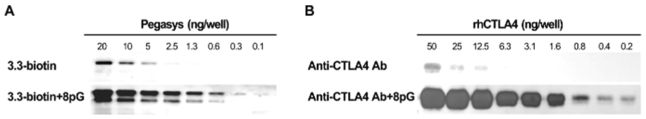

Figure 5. Western blot tests performed in the presence and absence of 8pG. Serial diluted (A) Pegasys and (B) rhCTLA4 were separated in a 10% reducing gel by SDS-PAGE, transferred to NC membrane, and stained with 3.3-biotin or anti-CTLA4 antibody which were mixed with or without 8pG as described in Experimental Section. Raw images are shown in Figure S3.

amplification of the 8pG group (3.3-biotin mixed with 8pG) for detecting Pegasys (20, 10, 5, 2.5 ng/well) was 2-fold, 3.6-fold, 5.6-fold, and 10-fold higher than that of the 3.3-biotin-alone group. The detection limit of 3.3-biotin-alone group was 1.3 ng/well. Attractively, after mixing 3.3-biotin with 8pG, the detection limit was improved to 0.1 ng/well. A similar result is also verified in another Western blot system using anti-CTLA4 to detect rhCTLA4. Figure 5B shows that the detection signal of each 8pG group (anti-CTLA4 antibody mixed with 8pG) was dramatically higher than that of the anti-CTLA4 antibody-alone groups. The detection limit of the 8pG group for detecting rhCTLA4 was enhanced to 0.2 ng/well, while the sensitivity of anti-CTLA4 antibody-alone group was 6.3 ng/well. These results demonstrate that 8pG could effectively improve the detection limit and sensitivity of Western blot systems.

Simple Mixing of Detection Antibodies with 8pG Enhances the Detection Signal of Flow Cytometry. Flow cytometry is an analytical technique for detecting specific surface proteins on cells, which has been routinely used in basic research and clinical studies. However, target molecules on the cell surface, if scarce, may not be effectively detected by this technique. The detection limit of current flow cytometry is restricted to at least 100 molecules of targeted protein per cell.[59] Although using antibody conjugated beads as signal enhancers could increase the amount of detection antibodies accumulating at the target molecules on cell surface, beads of size range from 50 to 100 nm[60,61] severely affected the granularity and size of targeted cells and resulted in inaccurate forward scatter (FSC) and side scatter (SSC) signals of the flow cytometer.[62−64]

8pG is a small-scale protein polymer with 60 kDa molecular weight, which makes its size smaller than 10 nm. We thus applied 8pG to improve the detection sensitivity of anti-PD-L1 antibody for detecting surface PD-L1 molecules on human PD-L1-positive HT-29 human colon cancer cells and human PD-L1-negative 3T3 mouse fibroblasts in flow cytometry. Figure 6A,B shows that the fluorescent signal of the 8pG group (anti-

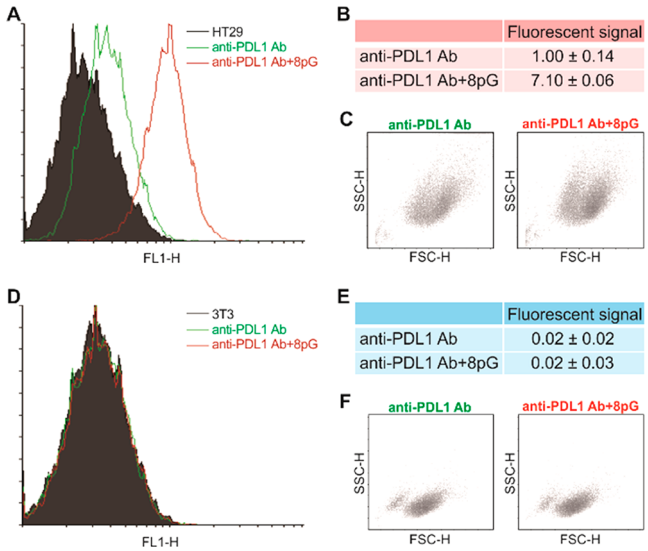

Figure 6. Flow cytometry tests performed in the presence and absence of 8pG. (A) PD-L1+ human colorectal cancer cells (HT-29) and (D) PD-L1-mouse fibroblasts (3T3) were analyzed by using an anti-PD-L1 antibody mixed with or without 8pG. The mean fluorescent signal ± SD of (B) HT29 cells and (E) 3T3 cells in each group was calculated by Flowing Software. The FSC/SSC distribution of (C) HT29 cells and (F) 3T3 cells in each group.

PD-L1 antibody mixed with 8pG) for detecting surface PD-L1 on HT 29 cells was 7.1-fold higher than that of the anti-PDL1 antibody-alone group. Mixing anti-PD-L1 antibody with 8pG did not increase the fluorescent background signal on PD-L1-negative 3T3 cells (Figure 6D,E). Importantly, Figure 6C,F shows that the FSC/SSC signal distribution of HT-29 and 3T3 cells in the 8pG groups were similar to the antibody-alone groups. These results demonstrate that binding of antibody/8pG complexes to target molecules on the cell surface can dramatically improve the detection sensitivity of flow cytometry systems without changing the granularity and size of the target cells

CONCLUSION

In this study, we exhibited an eight tandemly repeated protein G C2 domain polymer, 8pG, which can specifically trap the Fc region of IgG type antibodies from various species without interfering with the antigen binding ability of the Fab region. Simply mixing 8pG with detection antibodies can, in one step, generate a detection antibody/8pG complex which can dramatically increase the accumulation of detection antibodies on given antigen sites, thus enhancing the detection signal in ELISA, Western blot, and flow cytometry systems. Moreover, 8pG can be combined with other signal amplification strategies, such as detection antibody linked with photo-electrochemical enzymes or photoelectric materials, to develop

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ultrasensitive immunoassays. Collectively, we believe that 8pG is a powerful tool to universally amplify the detection signal and to improve the detection limit and sensitivity of various immunoassays applied in clinical diagnosis and biomedical studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b01077.

Conventional and 8pG-based anti-PEG ELISAs in BSA-blocked and BCS-blocked microplates, raw images of the Western blot, and position models of antibody/8pG complex (PDF)

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Author Contributions

Kuo-Hsiang Chuang, Michael Chen, and Yi-Jou Chen designed 8pG. Michael Chen and Yi-Jou Chen constructed the pLNCX-8pG-His plasmid. Yi-Jou Chen conducted the experiments whose data are presented in Figures 3,–6,. Chang-Hung Wang and Che-Yi Chen conducted the experiments whose data are shown in Figures 2, and 6. Hui-Lan Hsu conducted the experiments whose data are presented in Figures 3 and 4. Tian-Lu Cheng and Steve R. Roffler guided the anti-PEG antibody-associated experiments including the results presented in Figures 3–5. An-Pei Kao assisted in analyzing results and drawing Figure 1. Kuo-Hsiang Chuang, Yi-Jou Chen, Shyr-Yi Lin, and Jing-Jy Cheng formulated the manuscript and figures.

Notes

The authors declare no competing financial interest.

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Supporting Information

Simply mixing poly-protein G with detection antibodies enhances the detection limit and sensitivity of immunoassays

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Experimental section

The interference of bovine serum albumin (BSA) and bovine calf serum (BCS) blocking solutions on 8pG based ELISA. The PEG_{5K}-NH_2 serially diluted (0.69, 2, 6.2, 18.5, 55.5, 166 and 500 ng/well) in coating buffer (0.1 M $NaHCO_3$, pH = 9) was coated in Maxisorp 96-well microplates for 2 hours at 37 °C. The microplates were blocked with 3% (w/v) BSA or 3% (w/v) BCS in PBS overnight at 4 °C. Biotin-conjugated anti-PEG antibodies (2 μg/mL of 3.3-biotin) were mixed individually by a 1:1 volume ratio with 8pG at room temperature for 2 hours (the final concentrations of 3.3-biotin was 1 μg/mL). The 8pG/3.3-biotin mixtures were added to microplates and incubated at room temperature for 1 hour. After washing the microplates, streptavidin-HRP and ABTS were sequentially added and incubated in the microplates to detect PEG_{5K}-NH_2. Color development was measured at 405 nm by a microplate reader.

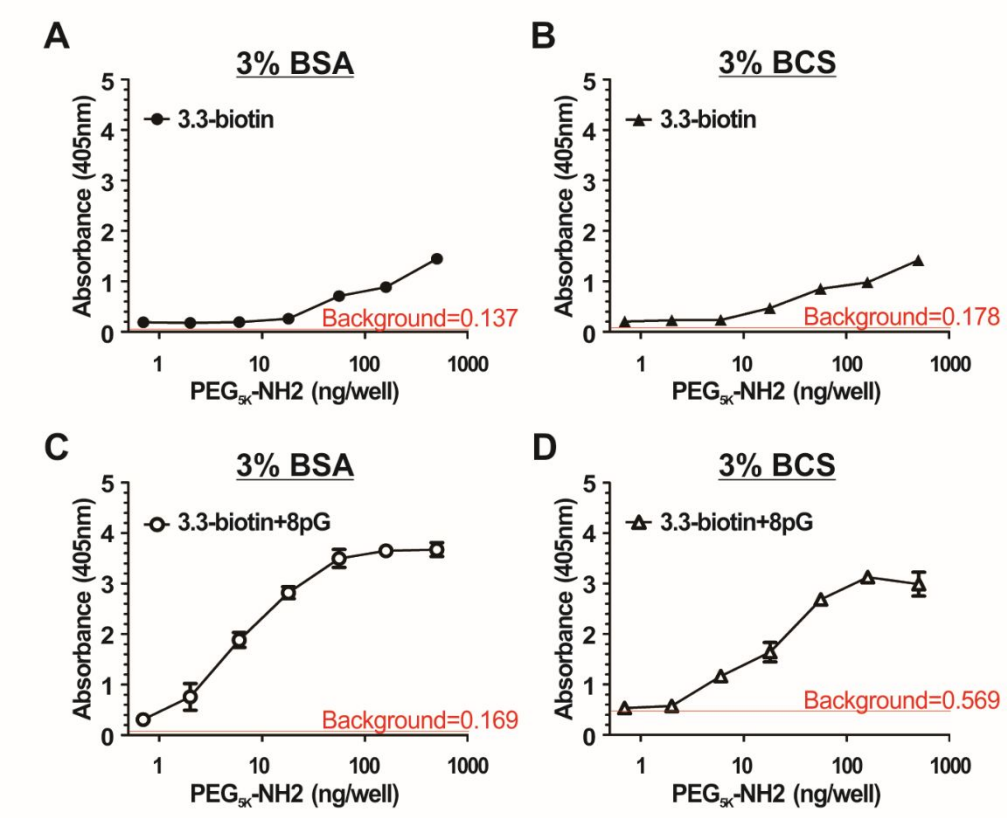

Figure S1. The interference of blocking reagents for the sensitivity and background value of 8pG based ELISAs. The microplates coated with different amount of PEG_{5K}-NH_2 (0.69, 2, 6.2, 18.5, 55.5, 166 and 500 ng/well) were blocked by (A)(C) 3% (w/v) BSA and (B)(D) 3% (w/v) BCS, respectively. Biotin-conjugated anti-PEG antibody (termed 3.3-biotin, 1μg/mL) mixing with or without 8pG was added to these microplates, followed by the sequential addition of streptavidin-HRP and ABTS substrate. The representative data from three independent experiments are shown. The red line indicates the background value of each group. Bar, SD.

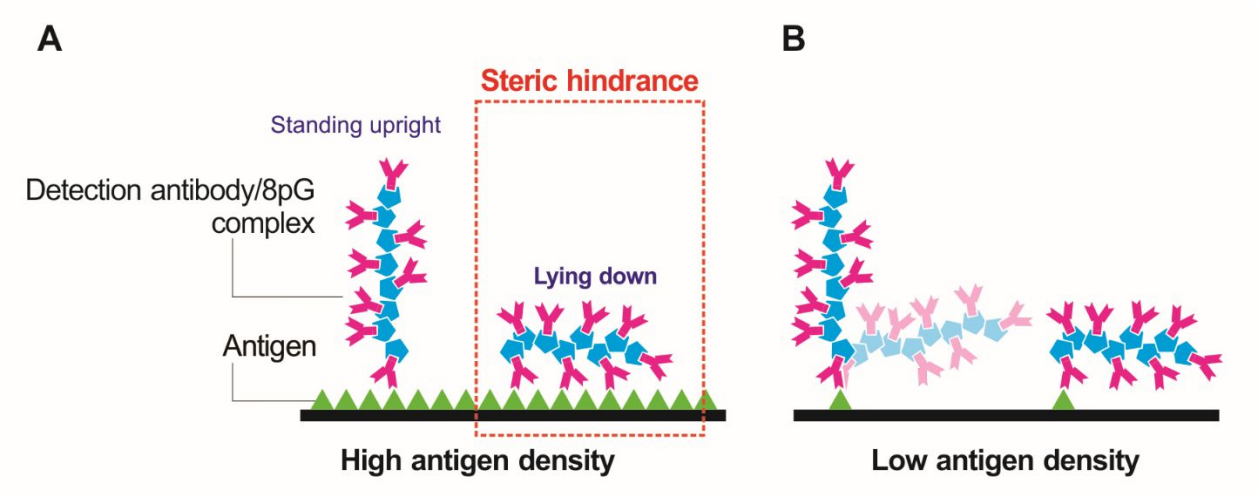

Figure S2. Models of antibody/8pG complexes "standing upright" or "lying down" on (A) high density or (B) low density antigens.

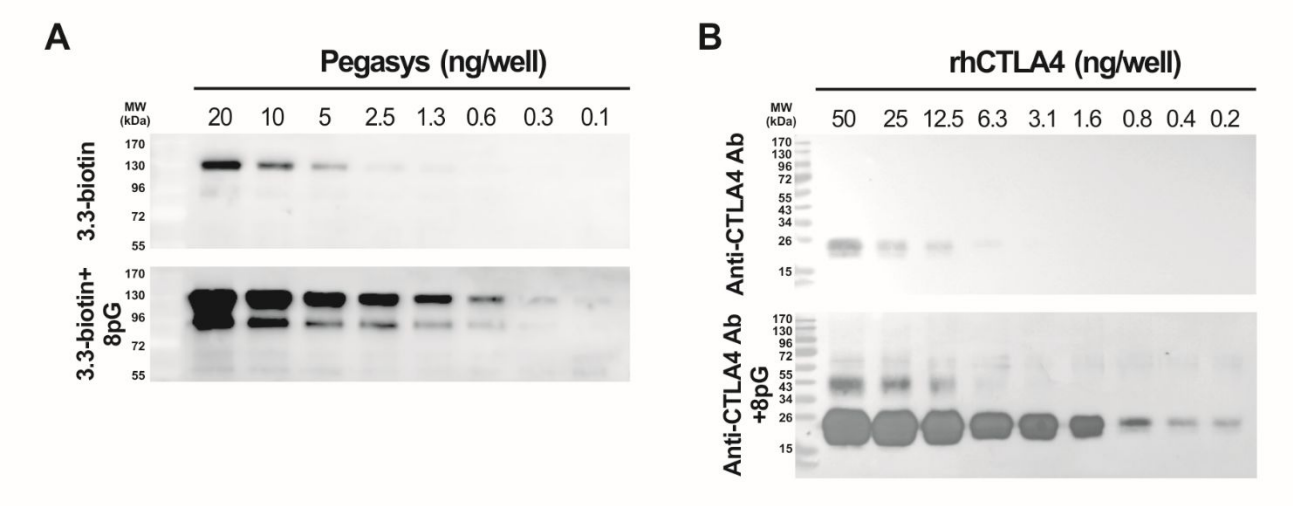

Figure S3. The raw images of Western blot data performed in the presence and absence of 8pG. Serial diluted (A) Pegasys and (B) rhCTLA4 were electrophoresed on a 10% reducing SDS PAGE, transferred to nitrocellulose membrane, and probed with 3.3-biotin or anti-CTLA4 antibody which were mixed with or without 8pG as described in Materials and Methods section.