Supporting Information

Solid-Phase Bioconjugation of Heterobifunctional Adaptors for Versatile Assembly of Bispecific Targeting Ligands

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MATERIALS

Monomeric avidin kit, N-Hydroxysulfosuccinimide (sulfo-NHS), sulfo-NHS-acetate, recombinant Protein L (PrL), recombinant Protein A (PrA), biotinylated horseradish peroxidase (HRP-biotin), Cell Lysis buffer, and 1-Step Turbo TMB-ELISA Substrate were purchased from Pierce Thermo Fisher Scientific (Rockford, IL)**.** Protein assay and PAGE supplies were purchased from Bio-Rad Laboratories (Hercules, CA). N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), human IgG Agarose, bovine serum albumin (BSA), and rabbit anti-androgen receptor antibody were purchased from Sigma Aldrich (St.Louis, MO). Biotin-PEG-NH_2 (10kDa) was purchased from NANOCS INC. (Boston, MA). Streptavidin (SA), biotinylated quantum dots (QD605-biotin), recombinant Protein G (PrG), and cell culture supplies were obtained from Life Technologies (now part of Thermo Fisher Scientific, Waltham, MA). Mouse anti-human CD3 antibody was purchased from BioLegend (San Diego, CA). Rabbit anti-Her2 antibody was purchased from EMD Millipore Chemicals (Philadelphia, PA). FITC-labeled goat anti-Protein A antibody was purchased from GeneTex (Irvine, CA). CytoTox 96 Non-Radioactive cytotoxicity assay and Cell Titer-Blue reagent were obtained from Promega (Madison, WI). SKBR3 and LNCap cell lines were obtained from ATCC. Human Peripheral Blood Mononuclear cells (PBMCs from healthy donors who are negative for bloodborne pathogens HIV-1 and 2, Hepatitis B, Hepatitis C, and HTLV) were obtained from Astarte Biologics (Redmond, WA).

METHODS

Mono-PEGylation of PrA(G,L) on a monomeric avidin column. Monomeric avidin column was washed with 10ml deionized water (DI H_2O) and incubated with 2ml sulfo-NHS-acetate (10mg/ml in pH 9.3 sodium bicarbonate buffer) for 1 hour at room temperature. Following washing with 30ml DI H_2O, 0.25mg (200µl) of biotin-PEG-NH_2 in water was loaded onto the column and incubated for 2 hours at room temperature. Free biotin-PEG-NH_2 was removed by washing with 30ml DI H_2O.

PrA(G,L) molecules (1mg/ml in PBS) were activated by EDC and sulfo-NHS (at 1:100:100 protein:EDC:sulfo-NHS molar ratio) for 15 min, then passed through a NAP-5 desalting column to remove excess EDC and sulfo-NHS**.** Activated PrA(G,L) were added to a monomeric avidin column at 6:1 biotin-PEG-NH_2 to PrA(G,L) molar ratio and incubated for 2 hours at room temperature. Following washing, PrA(G,L)-PEG-biotin conjugates were eluted from the column using 2mM D-biotin. Characterization of bioconjugation products was performed with 10% SDS-PAGE with Coomassie blue staining. Band density was measured using ImageJ. Purity of each conjugate was calculated as the ratio of a corresponding band density to the total density of all bands in one lane.

Assembly of heterobifunctional PrA(G,L)-PEG-SA adaptors on Human IgG-Agarose. A 2-ml column was prepared using 4ml of the IgG-agarose suspension, equilibrated with 0.01M pH7.2 PBS buffer, and blocked with 3% BSA-PBS. PrA(G,L)-PEG-biotin conjugates (1mg/mL, 0.5ml in PBS) were added to a column and incubated for 2 hours to allow PrA(G,L) binding to human IgG immobilized on agarose beads. After washing with 10ml PBS, 2mg streptavidin was loaded onto column and incubated for 2 hours at room temperature. Excess unbound SA was removed by washing with 10ml PBS. Then, final product was eluted with 6ml pH2.4 buffer composed of 0.1M Glycine and 0.15M sodium chloride. Characterization was performed using gel electrophoresis on a 0.5% agarose / 2.5% polyacrylamide hybrid gel[1] with Coomassie blue staining. Band density was measured using ImageJ. Purity of each conjugate was calculated as the ratio of a corresponding band density to the total density of all bands in one lane.

Characterization of PrA (G,L)-PEG-SA adaptor binding functionality. Serially diluted human IgG in a concentration range 0-250ng/ml was loaded in 96-well microtiter plates at 50µl/well in triplicates and incubated overnight at 4°C. Plates were further blocked with 3%BSA in 1x TBS buffer overnight. Then, 100µl 0.4nM PrA-PEG-SA, 0.6nM PrG-PEG-SA, or 0.4nM PrL-PEG-SA adaptors were added to each well and incubated for 2 hours at room temperature. A mixture of PrA(G,L), biotin-PEG-NH_2, and SA at the

same concentration was used as control. After washing with 1xTBS/0.05% Tween-20 for 4 times at 10 min each, 100µl biotin-HRP at 1:500 dilution was added and incubated for 2 hours at room temperature. Detection of surface-bound HRP was performed by incubating with 100µL/well soluble Turbo TMB-ELISA substrate for 5 min, then quenching reaction with 100µl/well Stop solution and measuring absorbance at 450nm using TECAN Infinite M200 plate reader.

Assembly of bispecific CD3xHer2 targeting ligands using PrA-PEG-SA adaptors. Biotinylation of anti-CD3 antibodies was done following previously published procedure.[1] Briefly, antibodies were reduced with 20mM DTT for 90 min and then reacted with 20x molar excess of maleimide-PEG2-biotin, which introduced a biotin residue to the hinge region. Assembly of CD3xHer2 ligands was performed in two steps. First, PrA-PEG-SA and anti-Her2 antibodies were mixed at 1:1 molar ratio and incubated for 2 hours at room temperature, followed by blocking of excess PrA binding sites with 5-fold excess of human IgG. Then, biotinylated anti-CD3 antibodies were added to pre-assembled Her2/PrA-PEG-SA constructs at 1:1 molar ratio and incubated for 1 hour at room temperature.

Characterization of target-binding functionality of CD3xHer2 bispecific ligands. Binding functionality of bispecific CD3xHer2 ligands was tested via specific labeling of CD3-positive human Peripheral Blood Mononuclear cells (PBMCs) and Her2-positive human breast cancer SKBR3 cells, analyzed with flow cytometry. Cells ($1x10^6$) were washed with PBS, spun down at 2000 rpm for 5 min, and incubated with 100µl 20nM bispecific CD3xHer2 ligands for 2 hour at 4^0C. Following washing with PBS, cell-bound ligands were labeled with 100µl 0.1mg/ml FITC-tagged anti-Protein A antibodies for 1 hour at 4^0C. Controls were assembled without anti-CD3 antibody for PBMCs and anti-Her2 antibody for SKBR3 cells. PBMCs and SKBR3 cells without treatment were used as a reference. Flow cytometry was performed with FACScan in Flow Cytometry Core Lab at the University of Washington.

Evaluation of CD3xHer2 bispecific antibody for T-cell based cell lysis. Human PBMCs were cultured in RPMI medium supplemented with 10% FCS and 100U/ml IL-2 overnight. SKBR3 cells were seeded in a 96-well plate at a density of 5,000 cells per well and grown overnight in RPMI medium supplemented with 10% FCS. For cell lysis experiment, 50µl of PBMC suspension was added to each SKBR3 well at an effector (PBMCs) to target (SKBR3) ratios of 25, 5, and 1 in triplicates. Then, bispecific CD3xHer2 antibody conjugates (1.50µL, 40nM) or equal amount of control (simple mixture of anti-CD3 and anti-Her2 antibodies) were added and incubated for 20 hours at 37°C. Additionally, five assay controls were included: (i) effector cell spontaneous LDH release, (ii) target cell spontaneous LDH release, (iii) target

cell maximum LDH release, (iv) volume correction, and (v) culture medium background. Aliquots (50µl) from each well were transferred to a new 96-well flat–bottom plate and incubated with 50µl LDH assay substrate for 30 minutes. Stop solution (50µl) was added into each well to quench reaction, and absorbance at 490nm was recorded with TECAN Infinite M200 plate reader. T cell-mediated lysis of SKBR3 cells was calculated using following formula:

$$\%\ \mathbf{cytotoxicity} = \frac{\mathbf{Experimental - effector\ spontanous - target\ spontanous}}{\mathbf{Target\ marximum - target\ spontanous}}\ \mathbf{X100}$$

1-step immunofluorescence cell staining with self-assembled antibody/quantum dots. Individual anti-androgen receptor (AR) antibodies were assembled with biotinylated red quantum dots (QD605-biotin, emission peak at 605nm) via PrA-PEG-SA adaptors by mixing antibodies, adaptors, and QDs at 1:1:1 molar ratio and incubating for 2 hours at room temperature. AR-positive human prostate cancer LNCap cells were used for this experiment. Cells were cultured and processed following procedure described elsewhere.[2] Briefly, cells were grown in glass-bottom 24-well plates, fixed with 4% formaldehyde, and permeabilized with 2% dodecyltrimethylammonium chloride (DTAC). Following washing with TBS, cells were blocked with 1% casein / 3% BSA for 15 min and then incubated with either pre-assembled AR/PrA-PEG-SA/QD605 probes or a control lacking anti-AR antibody (i.e. PrA-PEG-SA/QD605) at 10nM final probe concentration for 2 hours at room temperature. Images were captured with IX-71 inverted fluorescence microscope (Olympus) using a true-color charge-coupled device (QColor5, Olympus), X100 oil-immersion objective (NA 1.40, Olympus), and a wide UV filter cube (330-385 nm band-pass excitation, 420nm long-pass emission, Olympus).

1-step ELISA with self-assembled antibody/HRP. Individual anti-Her2 antibodies were assembled with biotinylated enzyme molecules (horseradish peroxidase, HRP-biotin) via PrA-PEG-SA adaptors by mixing 0.2nmole of antibodies, adaptors, and HRP at 1:1:1 molar ratio in 0.5ml PBS buffer and incubating for 2 hours at room temperature, yielding αHer2/PrA-PEG-SA/HRP stock solution. SKBR3 cell lysate was prepared using Pierce Cell Lysis buffer, and total protein concentration was determined with Bio-Rad Protein Assay. Serially diluted cell lysate was coated onto a 96-well microtiter plate overnight at 4^0C. Following washing, cells were blocked with 3% BSA for 3 hours at room temperature and then incubated with either pre-assembled αHer2/PrA-PEG-SA/HRP probes at 1:100 dilution from stock preparation or a control (simple 1:1 mixture of anti-Her2 antibody and biotinylated HRP) at equivalent antibody

concentration. Following washing, soluble Turbo TMB-ELISA substrate was added for colorimetric quantitation of HRP abundance. The absorbance at 450nm was measured with TECAN Infinite M200 plate reader.

Evaluation of PrA(G,L)-PEG-SA adaptor cytotoxicity. SKBR3 cells were seeded into a 96-well culture plate at 10,000 cells/well density and cultured in RPMI-1640 medium supplemented with 10% FBS at 37°C overnight. Then, serially diluted PrA(G,L)-PEG-SA adaptors were added to cells, and cells were further cultured for 48hours. Cell viability was tested with Cell Titer-Blue Reagent: 20µl/well reagent solution was added to cells and incubated for 2 hours. Fluorescent signal at $560_{Ex}/590_{Em}$ was measured with TECAN Infinite M200 plate reader.

REFERENCES

(1) Liu, H. Y., and Gao, X. (2011) Engineering monovalent quantum dot-antibody bioconjugates with a hybrid gel system. *Bioconjug. Chem. 22*, 510-517.

(2) Zrazhevskiy, P., True, L. D., and Gao, X. (2013) Multicolor multicycle molecular profiling with quantum dots for single-cell analysis. *Nat. Protoc. 8*, 1852-1869.

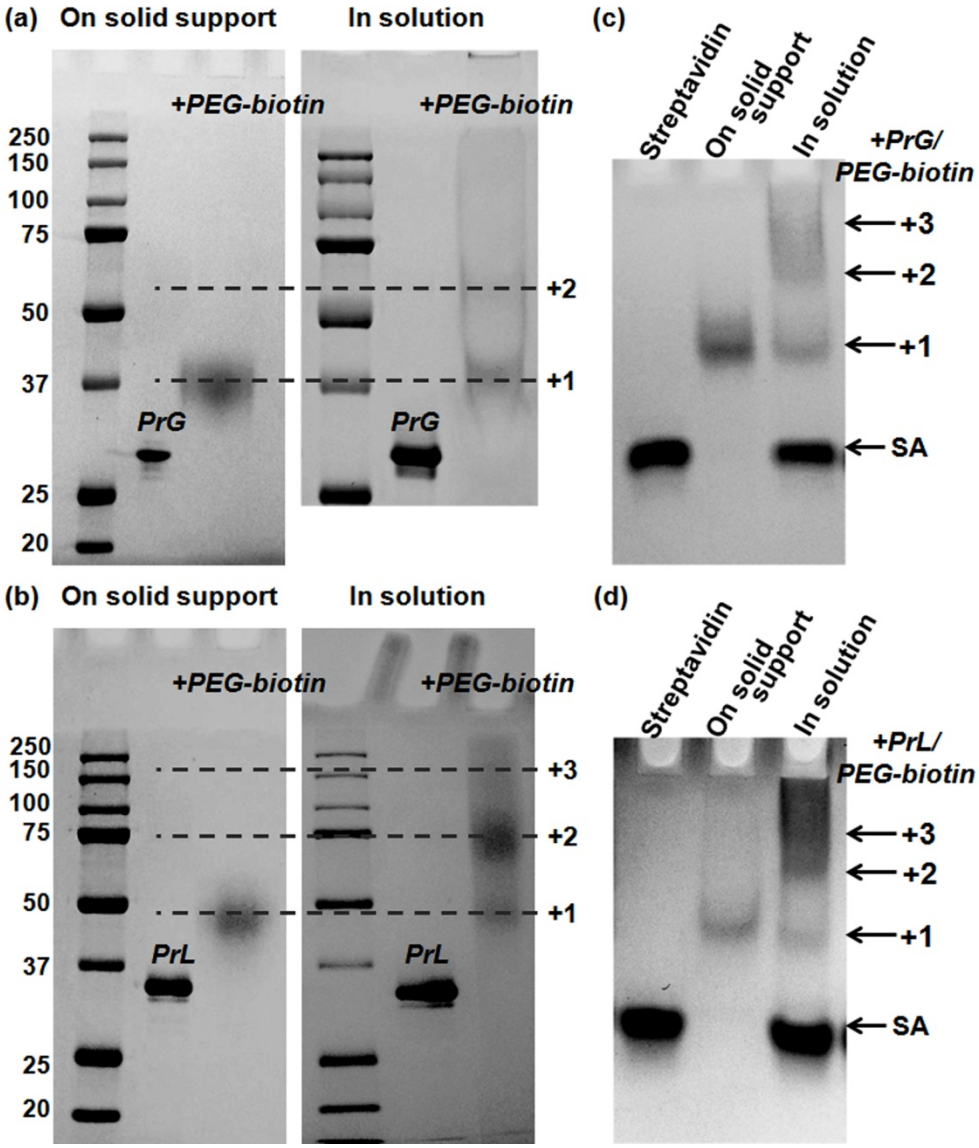

Figure S1. Characterization of heterobifunctional adaptor assembly on a solid support and in solution. Conjugation of PrG (a) and PrL (b) to biotin-PEG-NH_2 was performed on a monomeric avidin resin solid support and in solution. SDS-PAGE characterization of products reveals high purity of mono-PEGylated conjugates produced via solid-phase bioconjugation, in contrast to a heterogeneous mixture of PrL and PrG with 1, 2, 3 PEG molecules (along with large aggregates) produced by bioconjugation in solution. Molecular weight reference is shown in kDa. Further assembly of PrG-PEG-biotin (c) and PrL-PEG-biotin (d) with SA was performed on an IgG agarose solid support and in solution. Characterization with hybrid gel demonstrates 1:1:1 adaptor stoichiometry achieved with solid-phase procedure, whereas mixed SA conjugates with 0, 1, 2, 3+ PrA-PEG-biotin and PrL-PEG-biotin are produced via assembly in solution.

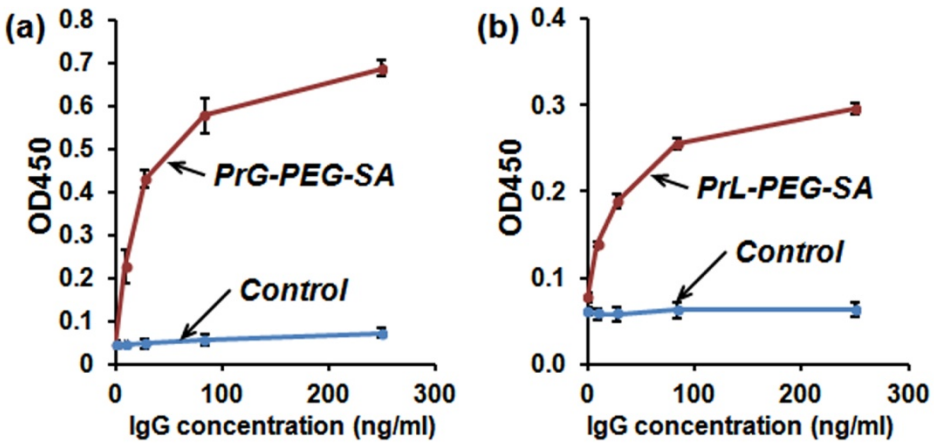

Figure S2. Characterization of adaptor functionality with ELISA. Immobilized IgGs were incubated with either PrG-PEG-SA (a) or PrL-PEG-SA (b), then labeled with biotin-HRP via binding to available SA sites on IgG-bound adaptors. IgG concentration-dependent signal detected with PrG-PEG-SA and PrL-PEG-SA adaptors confirms preserved functionality of both IgG-binding PrG and PrL blocks as well as biotin-binding SA block, whereas only background signal detected in control (simple mixture of adaptor blocks) shows lack of non-specific binding.

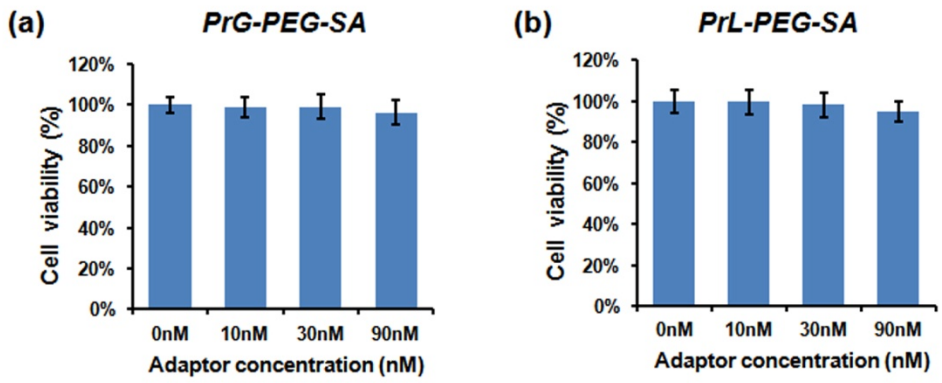

Figure S3. Cytotoxicity evaluation of heterobifunctional molecular adaptors. PrG-PEG-SA (a) and PrL-PEG-SA (b) adaptors at varying concentrations were added to SKBR3 cells cultured in a 96-well culture plate and incubated for 48 hours at 37°C. Cell viability was quantified with Cell Titer-Blue assay. Notably, adaptors induced no cytotoxicity in measured concentration range up to 90 nM, thus demonstrating compatibility with live cell assays.