Supporting Information

Structural and Functional Characterization of a Hole-Hole Homodimer Variant in a "Knob-Into-Hole" Bispecific Antibody

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This supporting information provides a detailed description of the materials and methods used for size-exclusion chromatography, native MS, negative-staining electron microscopy, the antigen binding assay to measure the binding of hole HD with the antigen peptide, AND.

Contents

Size Exclusion Chromatography

SEC was performed on an Agilent 1200 HPLC (Agilent Technologies, Santa Clara, CA) with a TSK G3000SW$_{XL}$ column, 7.8 × 300 mm (Tosoh Biosciences, King of Prussia, PA). The mobile phase was 0.2 M potassium phosphate buffer (pH 6.2) containing 0.25 M potassium chloride. Each sample was diluted to 1 mg/mL with the mobile phase. The protein load for each injection was 50 µg. The separation was conducted at ambient temperature with a flow rate of 0.5 mL/min. The column effluent was monitored at 280 nm UV wavelength.

Intact Mass Analysis by Native Mass Spectrometry

For the native MS experiment, 25–50 µg of the hole-hole homodimer samples were buffer-exchanged using Micro Bio-Spin TM 6 columns, prepackaged in Tris buffer (Bio-Rad Laboratories, Inc., Hercules, CA). The column was first centrifuged at 3300 rpm at 4 °C to flush the Tris buffer and then rinsed five times in 100 mM ammonium acetate (Sigma-Aldrich Corp., St. Louis, MO) by loading the column with 0.5 mL of buffer and centrifuging for 1 min at 3300 rpm at 4 °C. The collection tube was emptied after each spin. The column was then placed in a new collection Eppendorf tube, and 25–50 µg of sample were added on the resin at the center of the column. The column was then centrifuged for 10 min at 2000 rpm at 4 °C. The buffer-exchanged sample was collected in the Eppendorf tubes.

Samples were directly infused into an Exactive Plus extended mass range (EMR) Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA) via nanospray ionization using a Triversa TM Nanomate (Advion, Inc., Ithaca, NY). The instrument was set in EMR MS mode for intact mass analysis. The IgG samples were analyzed under the following acquisition parameters: spray gas pressure, 1.0 psi; spray voltage, 1.50 kV; Capillary temperature, 250 °C; S-lens RF level, 100; Scan rage, 1000 to 10000 m/z; Desolvation, in-source CID 120 ev, collision energy, 0; Resolution, 17500 at m/z 200; Polarity, positive; Microscans, 10; AGC targe, 3e6; Maximum injection time, 50; Averaging, 100; Source DC offset, 25V; Injection Flatapole DC, 8V; Inter Flatapole lens, 7V; Bent Flatapole DC, 6V; Transfer multipole DC tune offset, 0V; C-Trap entrance lens tune offset, 0V; Trapping gas pressure setting, 5. Spectra were visualized using Thermo Xcalibur Qual Browser then annotated manually. Mass spectrometric data was analyzed using Protein Deconvolution v4.0 software (Thermo Scientific).

Negative-Staining Electron Microscopy (NS EM)

Conventional cryo-electron microscopy (cryo-EM) is often the method of choice for studies of protein structure under physiological conditions because it avoids potential artifacts from fixatives and stains.[1] Still, cryo-EM studies of antibodies are challenging due to their small molecular mass (~150 kDa) and flexible structure.[2] Thus, we used an

optimized negative-staining (OpNS) protocol,[1,3] which was refined from the conventional negative staining protocol by using cryo-EM images as a control. OpNS EM images present a much higher image contrast than cryo-EM images with a reasonable resolution (1–3 nm) for visualizing the domains of each antibody. OpNS has been used to examine many proteins, such as antibodies,[4,5] GroEL,[6] and Glycyl-tRNA synthetase.[7] Through those studies OpNS has been validated as a general protocol.

The negative-staining specimens of antibody homodimers were prepared by using optimized negative-staining (OpNS) protocol as described previously.[1,3] Briefly, antibody samples were diluted to ~ 0.04 μg mL^{-1} with Dulbecco's phosphate buffered saline (DPBS). An aliquot (~ 4 μL) of diluted sample was then placed on an ultra-thin carbon-coated 200-mesh copper grid (CF200-Cu-UL, Electron Microscopy Sciences, Hatfield, PA, USA) that had been glow-discharged for 15 s. After 1 min incubation, the excess solution on grid was blotted with filter paper. The grid was then washed with water and stained with 1% (w/v) uranyl formate (pH ~4.5) before air-drying with nitrogen.[1,3]

Samples were examined by using a Zeiss Libra 120 Plus TEM (Carl Zeiss NTS) operated at 120 kV high tension. The micrographs were acquired under defocus between ~0.6 μm and ~0.9 μm. A dose of ~40-90 $e^-/Å^2$ using a Gatan UltraScan 4K X 4K CCD under a magnification of 80 kx (each pixel of the micrographs corresponds to 1.48 Å in specimens) was used. The contrast transfer function (CTF) of each micrograph was examined by ctffind3[8] and corrected by use of the SPIDER[9] software after the X-ray speckles were removed. Particles were then selected from the micrograph with a box size of 256 × 256 by use of boxer (EMAN[10] software). All particles were masked by a round mask generated from SPIDER after a Gaussian high-pass filtering. The reference-free class averages of particles were obtained by using refine2d (EMAN software) based on 2631 particles for the antibody sample stored at -70˚C.

Crystal structure of mouse IgG2 antibody (PDB entry 1IGT[11]) was superimposed on representative images of particles (Y- and X-shapes) to reflect the structural dynamics of the hole-hole homodimer. The two F_{ab} domains and two chains of F_c domains within 1IGT were rigid-body rotated and translated to obtain their best superimposition onto the representative particle images using Chimera.[12]

Antigen Binding Assay to Measure the Binding of Hole Homodimer with the Antigen Peptide

A 96-well high binding polystylen microtiter plate was incubated for 16-72 h at 4 °C with 100 μL of 3 μg/mL of Neutravidin in DPBS (-$Ca^{2+,}$ -Mg^{2+}). The plate was washed with wash buffer (1x PBS, pH 7.4, 0.05% Polysorbate 20) twice with auto program (6 times wash) using a BioTek 405 plate washer. The plate was blocked with 200 μL of blocking buffer (1x PBS, pH 7.4, 0.05% Polysorbate 20, 0.5% BSA) at 25 °C for 1-2 h. Then the plate was washed as above. 100 μL of biotinylated antigen peptide (2 μg/mL) in assay diluent (1x PBS, 0.05% Polysorbate 20, 0.5% BSA) was added to each well, and incubated at 25 °C for 1 h. While incubating the plate, experimental samples were diluted accordingly using assay diluent. After incubation with the antigen peptide, the plate was washed as described above, and 100 μL of the sample diluents were added to the plate. The sample diluents were incubated at 25 °C for 1 h. After washing, 100 μL of 3 ng/mL of goat anti-human-IgG (Fab')2 HRP (Jackson ImmunoResearch

Cat.No.109-036-097) in assay diluent was added and incubated at 25 °C for 1 h. The plate was washed after the incubation, and 100 µL of TMB substrate (SureBlue Reserve TMB Microwell Poroxidase substrate, KPL, cat. No. 53-00) was added and incubated until the color developed adequately. 100 µL of 0.6N sulfuric was added to quench the reaction. The plate was measured for the optical density (OD) values on the plate reader using two wavelengths, 450 nm for detection absorbance and 650 nm for reference absorbance. The data were analyzed with SoftMaxPro software by 4P analysis, and relative potency was calculated using the -70 °C hole homodimer as standard.

FcRn Affinity Chromatography to Evaluate the Hole-hole Homodimer's Fc Structural Integrity

FcRn affinity chromatography was performed on Thermo/Dionex Ultimate 3000 UHPLC system. The soluble extracellular domain of FcRn with His-Avi-Tag® associated with beta-2-microglubulin was immobilized onto POROS® streptavidin beads that were packed into a 300 µL column[13]. Mobile phase A was 20 mM MES(2-(N-morpholino)ethanesulfonic acid , 150 mM NaCl, pH 6.0, mobile phase B was 20 mM MES, 150 mM NaCl, pH 6.5 and mobile phase C was 20 mM Tris/HCl, 150 mM NaCl, pH 8.5. The flow rate was 0.25 mL/min. Samples were diluted to 1.0 mg/mL in mobile phase A. Approximately 30 µg of each sample was loaded onto the column, where it was eluted using the following pH gradient: 100% mobile phase A to 66% mobile phase B in 5 minutes; to 75 % B and 25% C in 5 minutes; to 15% B and 75% C in 20 minutes; to 100% C in 8 minutes; hold 100% C for 9 minutes; to 100% A in 2 minutes; hold 100% A for 12 minutes. The elution was monitored by UV absorption at 280 nm, and the pH was monitored using an on-line pH meter.

Figure S-1. Hydrophobic interaction chromatography (HIC) profile of the hole-hole homodimers

Hole-hole homodimer (HD) at different time points during storage at 2-8 °C, after buffer exchange to pH 7.5 from the first buffer exchange (pH 5.8 to pH 3.0) for 1.5, 3 and 18 h, compared with the hole-hole HD frozen control. (Note: the -70 °C controls were made from different batches, so the frozen control here is different from the ones in Figure 1.)

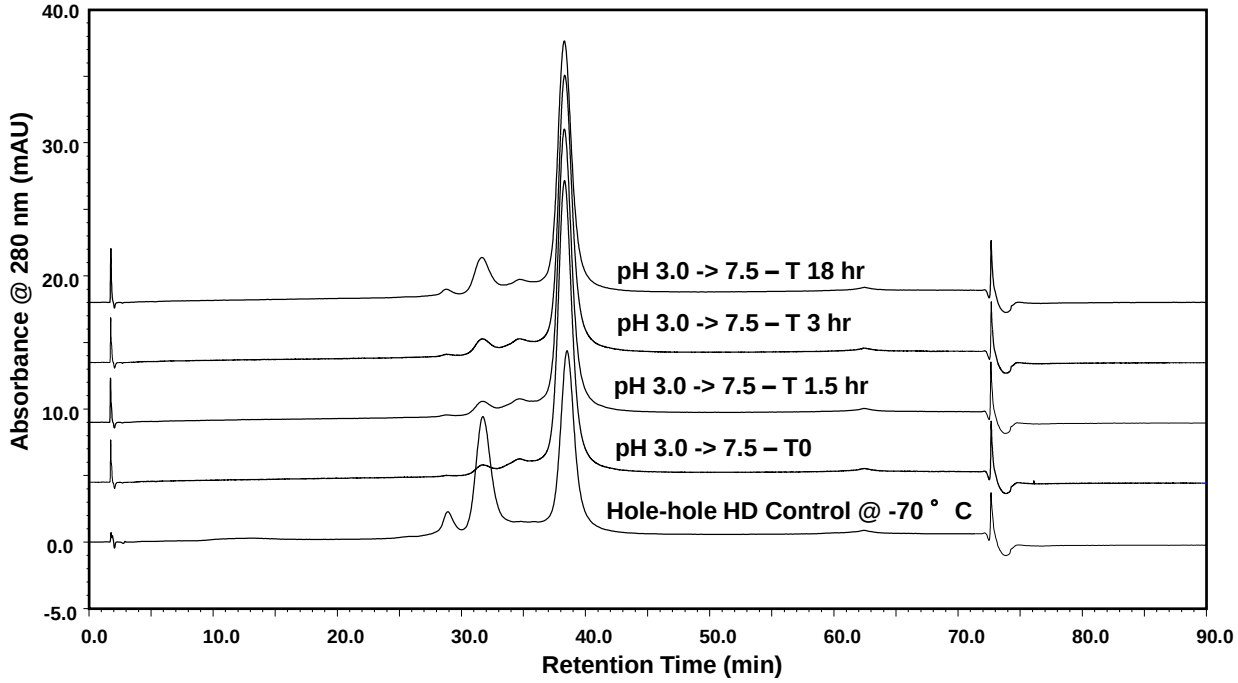


Figure S-2. SEC for the hole-hole homodimer (HD) stored under different conditions and lengths of time (The insert is the overlay).

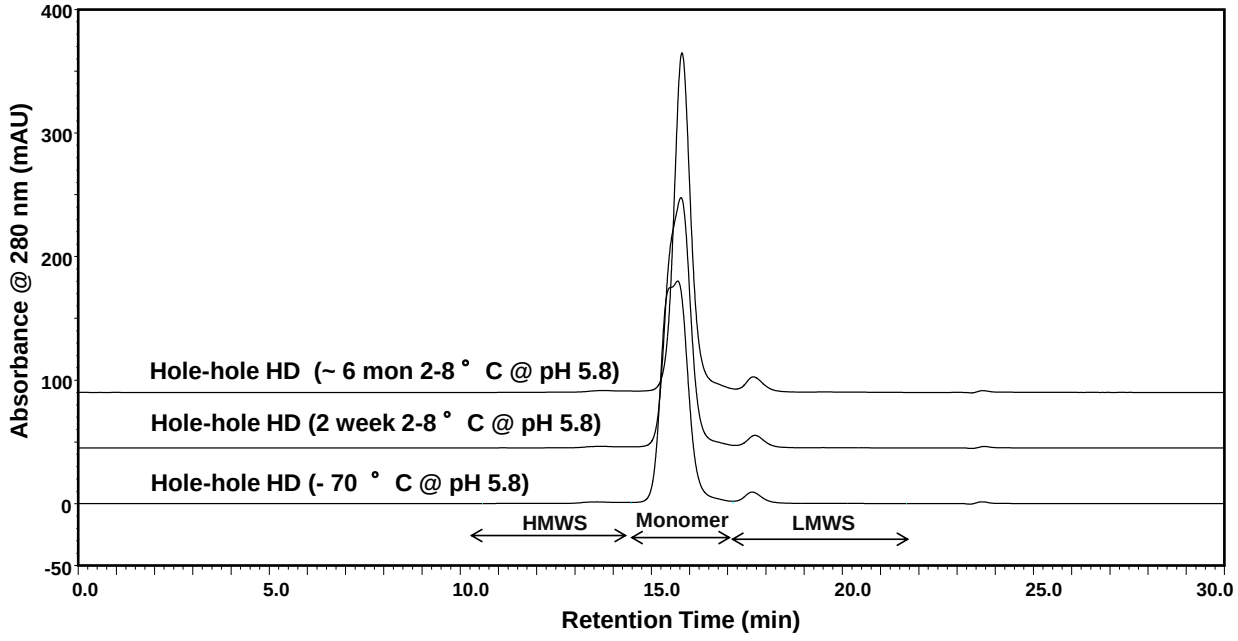
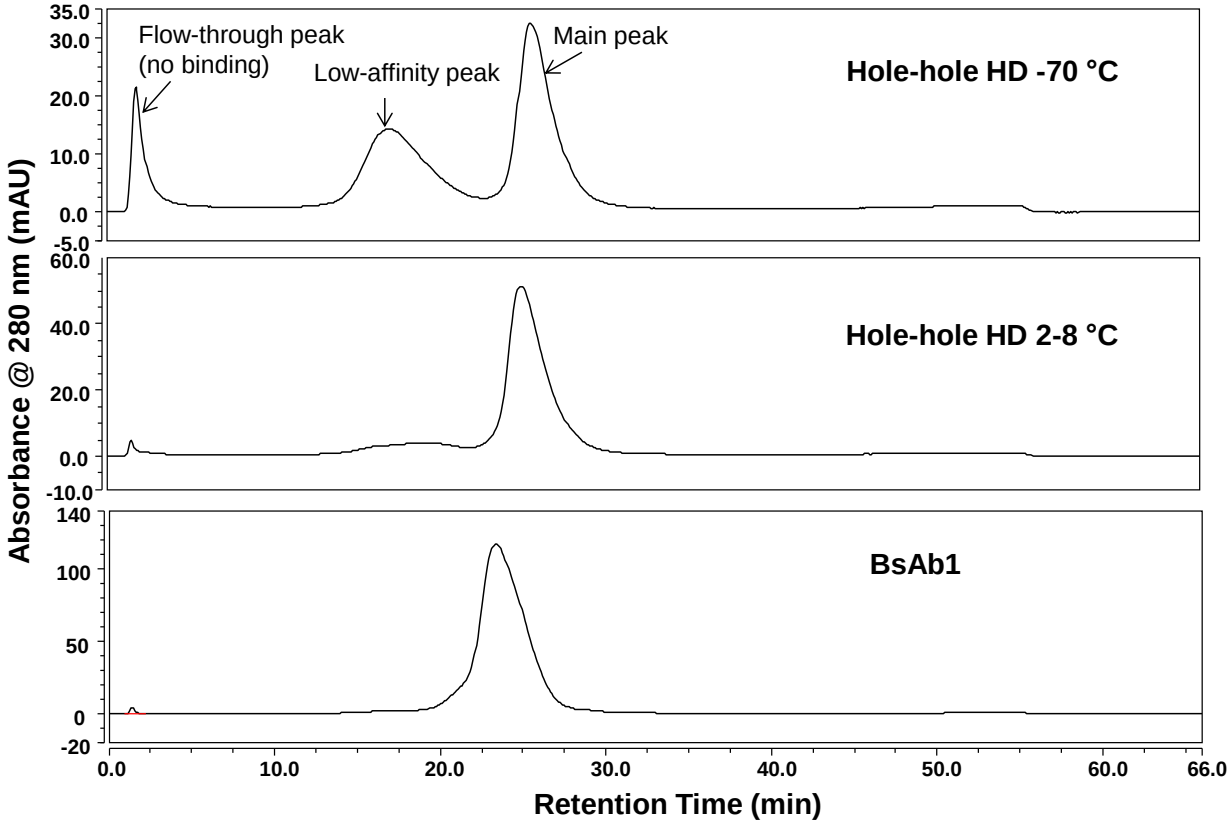


Figure S-3. FcRn Affinity Chromatography for the hole-hole homodimer (HD) stored at -70 °C and 2-8 °C, as well as the BsAb1 samples.

The hole-hole HD stored at -70 °C displayed three peaks, a flow-through peak (no binding), a low-affinity binding peak, and a main peak. The hole-hole HD stored at 2-8 °C displayed a main peak and a small peak with low-affinity binding. The BsAb1 displayed a single main peak. Note that the retention time is slightly different between the bispecific antibody and the hole-hole HD at 2-8 °C, possibly due to their slightly different Fc structure.



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