Characterization of the Native and Denatured Herceptin by ELISA and QCM using a High-Affinity Single Chain Fragment Variable (scFv) Recombinant Antibody

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Supporting material and methods:

S1. **Synthetic peptide (CSRWGGDGFYAMDY) conjugation to BSA.** Generally, the heavy chain CDR3 region of an antibody is unique to an antigen-specific antibody, and to no other antibody, and confers most of the antibody's antigen-binding specificity. The amino acids that make up the Herceptin heavy chain CDR3 region are SRWGGDGFYAMDY. A naturally occurring cysteine (C) precedes the SRWGGDGFYAMDY amino acids and was incorporated into the synthetic peptide to couple the peptide to carrier proteins (e.g. BSA) for phage selection purposes. The CSRWGGDGFYAMDY peptide was coupled to maleimide-activated BSA (Pierce, Cat. *No.* 77116) via the C-amino acid according to the manufacture's protocol. The BSA-conjugated CSRWGGDGFYAMDY peptide was then passed over a NAP-10 column (G. E. Healthcare, Cat. *No.* 17-0854-02) equilibrated with phosphate buffered saline (PBS) to separate BSA-conjugated CSRWGGDGFYAMDY from free peptide.

S2. **Phage recombinant antibody selection and ELISAs to detect and characterize scFv specific for Herceptin.** An *E. coli* TG1 glycerol stock containing the scFv DNA recombinant antibody library in pCANTAB5E was grown in 2xYT bacterial culture medium containing 2% glucose and 100 μg/ml of ampicillin (2xYTAG) and helper phage rescued with M13KO7 to

produce an active phage infection. Phage-displayed recombinant scFv antibodies produced during phage infection were PEG-precipitated and suspended in PBS then used for phage scFv selections. Immunotubes (Nunc, Cat. *No*. 444202) were coated with 1 mL of PBS containing 10 µg of BSA-conjugated CSRWGGDGFAMDY, then blocked with PBS containing 0.1% Tween 20 (PBS-T). Tween 20 was added to the PEG precipitated phage scFv library to a final concentration of 0.1%, then incubated for 30-60 minutes at room temperature as a blocking agent. The blocked phage-displayed recombinant scFv library was applied to the BSA-CSRWGGDGFAMDY coated tubes and incubated for 2-3 hours at room temperature. Tubes were emptied then washed 10 times with PBS-T to remove phage-displayed scFv not bound to BSA-CSRWGGDGFYAMDY. One mL of 10 µg/ mL of Herceptin diluted in PBS-T was added to phage-scFv on BSA-CSRWGGDGFYAMDY coated tubes, and incubated for 1 hour at room temperature with shaking at 100 rpm to competitively elute phage scFv bound to the SRWGGDGFYAMDY peptide. Eluted phage scFv were used to infect log phase grown *E. coli* TG1 cells. Infected *E. coli* TG1 cells were plated onto 2xYTAG agar plates and grown overnight at 30°C. Cells on 2xYTAG plates were M13KO7 helper phage rescued to obtain phage-displayed scFv for a second round of phage selections on BSA- CSRWGGDGFYAMDY. Phage-displayed scFv were PEG-precipitated and used for a second round of selection as described above, with the following modifications. Immunotubes were coated with BSA-CSRWGGDGFYAMDY at 5 µg/ mL PBS, and phage scFv bound to SRWGGDGFYAMDY were eluted with Herceptin diluted to 50 µg/ mL PBS-T. Bacterial colonies stemming from 2 rounds of phage selection were picked from 2xYTAG agar plates to wells of 384 well microtiter plates containing 2xYT AI (100 µg/ml ampicillin, 1 mM IPTG) and induced to express soluble E-tagged scFv. Soluble E-tagged scFv were obtained from *E. coli* periplasmic extract according to the G.E. Healthcare

Recombinant Phage Antibody System (RPAS) Expression Module (Cat. *No.* 27-9401-01). ELISAs were used to detect E-tagged scFv bound to Herceptin. Nunc (Cat. *No.* 242757) 384 well microtiter plates were coated with Herceptin diluted to 10 µg/ mL PBS for 1 hour at room temperature then blocked with PBS-T. Twenty-five µL of periplasmic extract containing E-tagged scFv was added to microtiter wells, incubated for 1 or more hours at room temperature. Microtiter plates were washed 4-6 times with PBS-T to remove unbound scFv. Twenty-five µL of HRP-conjugated Anti-E tag antibody, diluted 1:8,000 in PBS-T, was added to each well then incubated for 1 hour at room temperature or overnight at 4-8 ^{o}C. Microtiter wells were washed 4-6 times with PBS-T after which 25 µL of ABTS [2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] and hydrogen peroxide was added to each well to produce a visible green color reaction for wells in which E-tagged scFv bound to Herceptin. *In lieu* of anti-E tag/HRP, streptavidin/HRP (Sigma, Cat. *No.* S5512) diluted to 0.4 µg/ mL PBS-T was used in ELISA to detect biotinylated scFv (Figure 2B) bound to Herceptin. A Biotek 800 ELxNB microtiter plate reader operating at 405 nm was used to determine absorbance readings.

S3. **Recombinant scFv purification and biotinylation.** Recombinant scFv were purified from *E. coli* periplasmic extract using the RPAS Purification Module (G.E. Healthcare, Cat. *No.* 17-1362-01) according to manufacturer's instructions with the following modification. Recombinant scFv were eluted using 0.1 M citric acid (pH 2.9) rather than 0.1 M glycine. The pH of the eluted scFv was adjusted to greater than 5 using NaOH, and stored at 4-8 ^{o}C. ScFv concentration was determined using a UV/Vis spectrophotometer operating at 280 nm. A 1 mg/ mL concentration of scFv was arbitrarily assumed to give an optical density reading of 1 at 280 nm. The molecular weight of scFv stemming from the phage antibody library is ~27 kDa. Prior to biotinylation, 1 M sodium bicarbonate (pH 8.3) was added to purified-scFv to achieve a final

concentration of 0.025 M- 0.1 M sodium bicarbonate. Biotinamidocaproate-NHS (Sigma, Cat. *No.* B-2643) diluted in dimethyl sulfoxide (DMSO) was added to scFv in at a 2, 3 or 4 moles of biotin: 1 mole scFv ratio. The scFv were biotinylated overnight at 4-8 ^{o}C. The biotinylation reaction was terminated with the addition of 1/10th volume of 1 M Tris, pH 7.6. Biotinylated scFv were used as is for ELISA assays without removing any unincorporated biotin from biotinylated scFv preparations.

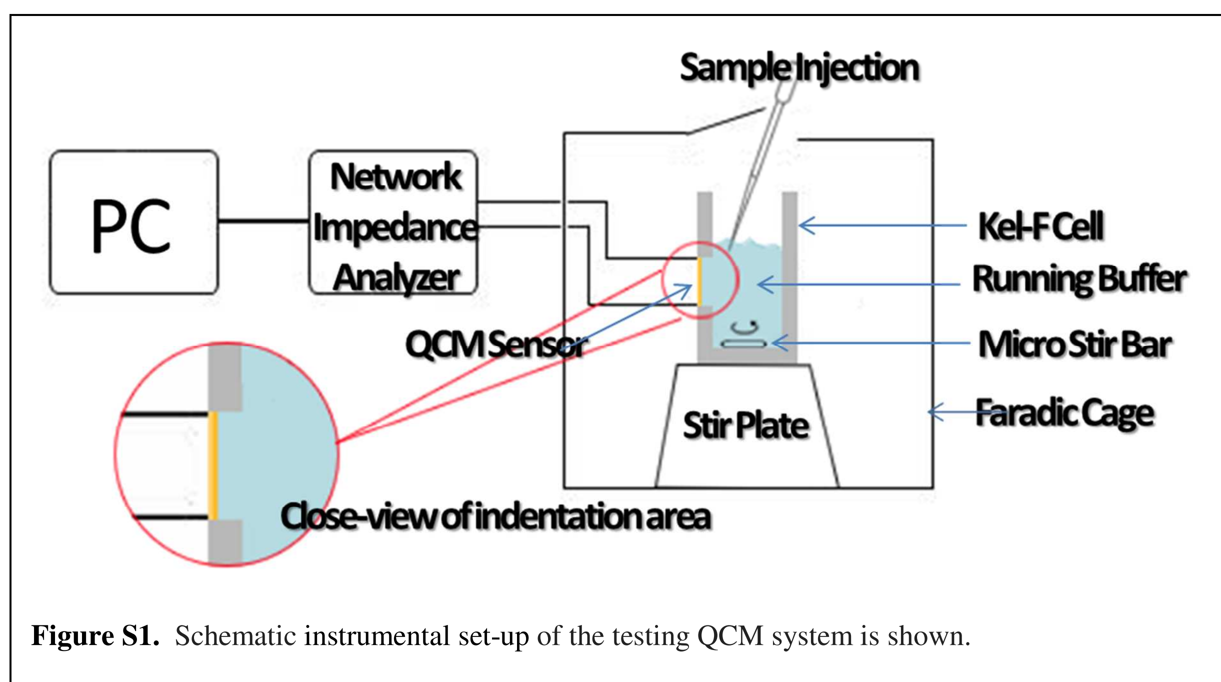

Figure S1. Schematic instrumental set-up of the testing QCM system is shown.

S4. Electrochemical Characterization of the 2B4-scFv modified QCM Au quartz transducer by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS).

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were used to characterize the 2B4 scFv-modified QCM gold surface. All experiments were carried out using a three-electrode system with a bare or modified gold electrode as the working electrode, a platinum wire as the counter electrode and a Ag/AgCl reference electrode (saturated KCl) incorporating a potentiostat/galvanostat (EG&G Par Model 2263). One mL of 0.1 M $NaClO_4$

containing 1 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) was used as a supporting electrolyte. The CV potential was scanned from -0.25 to 0.75 V at a scan rate of 50 mV/s. The EIS measurements were obtained by applying a 5 mV amplitude sine wave under DC bias at open circuit potential within a frequency range of 0.01 Hz to 100 KHz.

The two steps used to prepare the 2B4-scFv QCM sensor surface were as follows: 1) NeutrAvidin was first bound to the Au QCM by non-specific adsorption and 2) biotinylated 2B4-scFv was coupled to NeutrAvidin on the sensor surface. CV and EIS were used to monitor QCM sensor surface preparations and interactions between 2B4-scFv and therapeutic antibodies. Redox $[Fe(CN)^{3-}/Fe(CN)^{4-}]$ probe peaks, as an indication of sensor surface binding events, sequentially decreased upon NeutrAvidin, biotinylated 2B4-scFv and heat-denatured Herceptin surface deposition (Figure S2 A). Nyquist (semi-circles - Figure S2 B) and Bode plots (insert - Figure S2 B) demonstrated that the electron transfer resistance of the redox probe increased step-by-step upon sequential addition of NeutraAvidin, biotinylated 2B4-scFv and heat denatured Herceptin to the sensor surface.

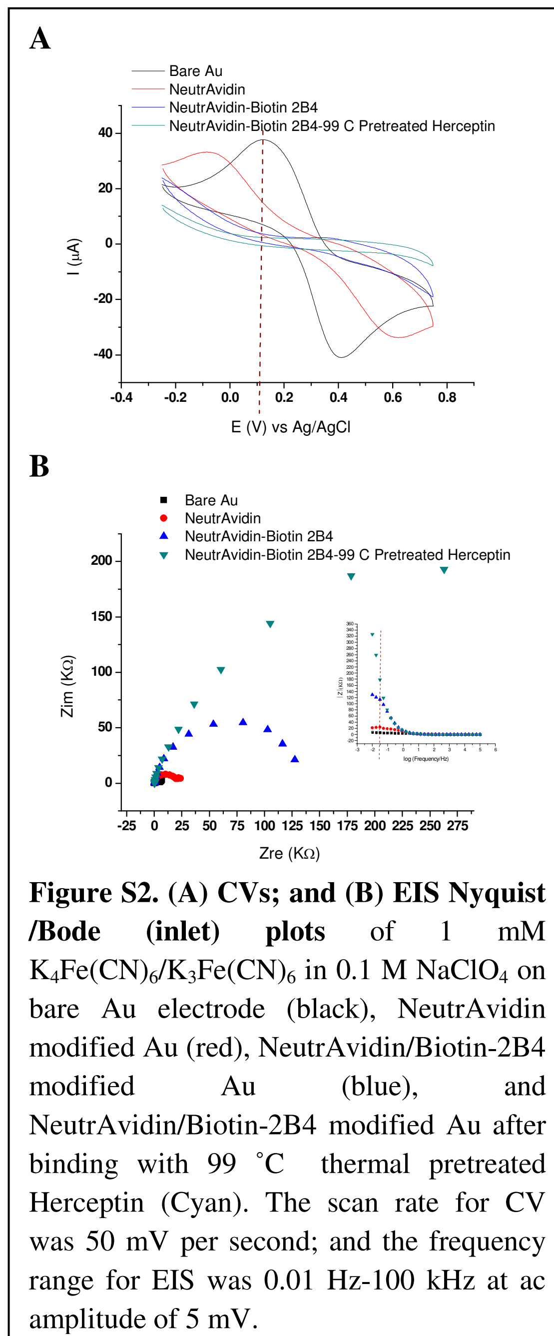

Figure S2. (A) CVs; and (B) EIS Nyquist /Bode (inlet) plots of 1 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ in 0.1 M $NaClO_4$ on bare Au electrode (black), NeutrAvidin modified Au (red), NeutrAvidin/Biotin-2B4 modified Au (blue), and NeutrAvidin/Biotin-2B4 modified Au after binding with 99 ˚C thermal pretreated Herceptin (Cyan). The scan rate for CV was 50 mV per second; and the frequency range for EIS was 0.01 Hz-100 kHz at ac amplitude of 5 mV.

Table S1. CV and EIS 2B4-scFv surface sensor changes.

Au electrode	CV	EIS
	I_{pc} (μA) at E ≈ 0.122 V	∣Z∣ (KΩ) at frequency ≈ 30 mHz
NeutrAvidin/Biotin-2B4 Herceptin Au	~2.4	~ 178.5
NeutrAvidin/Biotin-2B4 Au	~3.9	~ 113.6
NeutrAvidin Au	~ 15.0	~ 24.3
Bare Au	~ 37.8	~ 6.3

S5. UV-vis Characterization of Herceptin Self-association upon heating

UV–visible absorbance measurements were performed using a Cary 100 Bio UV–visible spectrophotometer at room temperature. The light path for UV–Vis spectra measurements was 1 cm. 147 μM Herceptin was diluted by biograde water to be 5 μM and sample heating conditions are similar to those shown in Figure 5B.

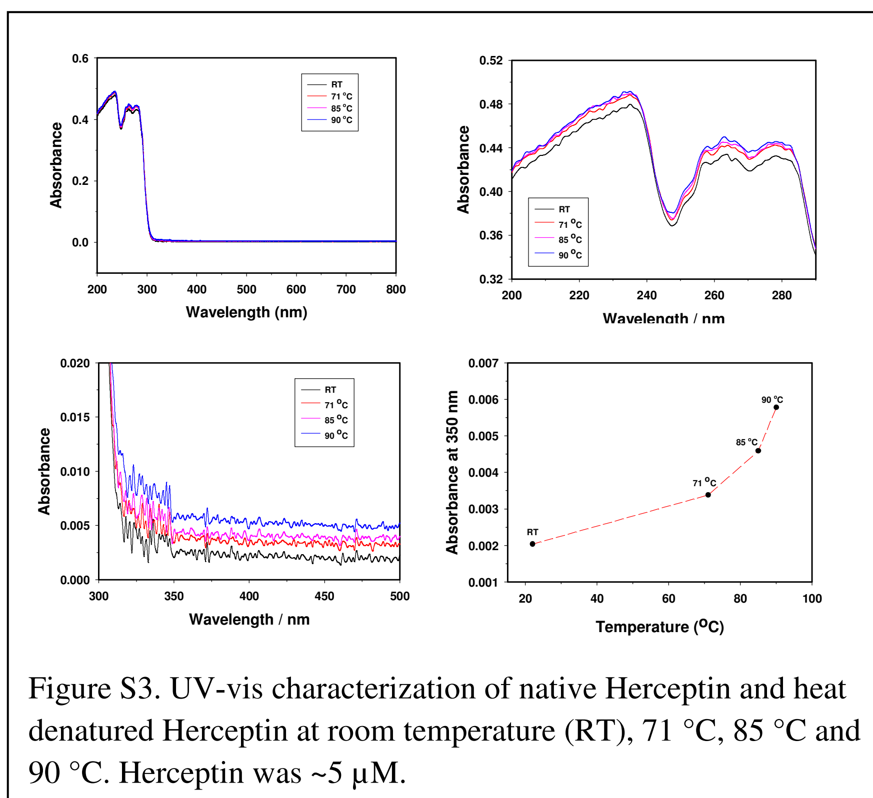

Figure S3. UV-vis characterization of native Herceptin and heat denatured Herceptin at room temperature (RT), 71 °C, 85 °C and 90 °C. Herceptin was ~5 μM.