Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

Self-Assembling Antibodies by Chemical Induction

*Qing Li, David Hapka, Hua Chen, Daniel A. Vallera, and Carston R. Wagner**

Supplementary Method

Construction of p13DD13CD3

We constructed anti-CD3-$DHFR^2$ fusion proteins based on the p13DD plasmid template previously made in our lab, which was derived from the pFLAG-ATS expression vector. The p13DD plasmid encodes a fusion protein containing a wild-type ecDHFR linked by a 13 amino acid linker (GLGGGGGLVPRGT) to a second copy of wild-type ecDHFR. Our construction scheme relied on the insertion of anti-CD3 scFv gene into the C-terminal of p13DD plasmid.

Firstly, the forward primer 5'-GAAATCCTCGAGCGTCGTGCGGGCGAAAA CCTGTATTTTCAGGGAATCGGTCTAGATAGATGAGCTCG-3' and the reverse primer 5'-CGAGCTCATCTATCTAGACCGATTCCCTGAAAATACAGGTTTTCGC CCGCACGACGCTCGAGGATTTC-3' were used to remove the stop codon and insert a 13 amino acid linker (AGENLYFQGIGLD) containing a TEV protease site between the end of the $DHFR_2$ gene and the XbaI restriction site by Quick Change^{TM} insertion mutagenesis.

Secondly, the anti-CD3 scFv was amplified from the plasmid Mo3.pGEMT.e, kindly provided by Dr. Daniel Vallera. The upstream primer 5'-GGTCTAGATGACATCCAGATGACCCAGACC-3' supplied the XbaI restriction site, and the downstream primer 5'-GAGCTCCTATGAGGAGACGGTGACGGT

GG-3' supplied the SacI restriction site.

Finally, both the p13DD13 and the amplified anti-CD3 scFv gene were sequentially digested for 4 hours at 37ºC with XbaI and then another 4 hours at 37ºC with SacI. Ligation of the double digested anti-CD3 scFv and p13DD13 fragments, mixed at a range of stoichiometries, with T4 DNA ligase (Promega, WI) for 18 hours at room temperature, followed by transformation into z-competent XL1-Blue *Escherichia coli* cells yielded colonies bearing the ligated plasmid, p13DD13CD3.1. This plasmid encodes a fusion protein containing two copies of ecDHFR linked by a 13 amino acid linker to the anti-CD3 scFv gene. The DNA sequence was verified (University of Minnesota, Advanced Genetic Analysis Center) to confirm the gene had no mutations.

Multimutagenesis of four cysteines in the two DHFRs of the p13DD13CD3

In the process of the protein preparation, the presence of the four cysteines in the two DHFRs of the anti-CD3-DHFR^2 would disturb the formation of the disulfide bonds in the anti-CD3 scFv. In order to mutate the four cysteines in the two DHFRs, the three primers 5'-GCCATCGCGGCGGCTGGTGACGTACCAGAAATCATGG-3', 5'-GCAGAACTCTCACAGCTATAGCTTTGAGATTCTGG–3', 5'-GCAGAACTC GCATAGCTATAGCTTCGAAATCCTCGAGCG-3' were used to obtain C85A C152S C257A C324S p13DD13CD3.2 by QuikChange® Multi Site-Directed Mutagenesis (Stratagene). The DNA sequence was verified (University of Minnesota, Advanced Genetic Analysis Center) to confirm the four cysteines mutations.

Protein expression, refolding and purification

Plasmid was transformed into the *Escherichia coli* strain BL21 (DE3) (Novagen). Bacteria were grown in 1L LB medium containing 100ug/ml carbenicillin in 4 flasks with shaking and aeration to an OD_{600} in the range of 0.6-0.8, whereupon isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the concentration of 1mM. Four hours after induction, the bacteria were harvested by centrifugation.

The cell paste was suspended and sonicated 8x30 seconds in the lysis buffer (50mM Tris, 50mM NaCl, 5mM EDTA, pH 8, 1mM PMSF), with at least 2 minutes between each repetition to allow the lysate to cool below 10 ºC. After centrifugation, the cell pellets were extracted and washed to obtain partial purified inclusion body (PPIB) with wash buffer (0.3% sodium deoxycholate, 5% Triton X-100, 10% glycerin, 50mM Tris, 50mM NaCl, 5mM EDTA, pH 8).

The proteins were refolded using sodium N-lauroyl-sarcosine (SLS) air oxidation method. Inclusion bodies were dissolved in 100mM Tris, 2.5% SLS, and incubated at room temperature with rapid stirring for 20 hours for air-oxidization of –SH group after adding 50uM of $CuSO_4$ to the solution. After removing detergent SLS by adding 6M urea and 10% 1-X8 resin (200-400 mesh, chloride form), the protein solution was diluted 40-fold with refolding buffer (50mM Tris, 0.5M L-arginine, 20% glycerin, 5mM EDTA, pH 8), then incubated at 4 ºC for 2 days.

After dialyzing first against Tris buffer (20mM, pH 9) and then equilibration buffer (10mM KH_2PO_4, 0.1mM EDTA, 0.5M KCl, pH 6), the protein solution was loaded onto a methotrexate affinity column that had been prepared with phosphate buffer

(50mM KH_2PO_4, 1mM EDTA, pH 6). The column was washed with high salt buffer (50mM KH_2PO_4, 1mM EDTA, 1M KCl, pH 6) until A_{260} and A_{280} of the flow-through were less than 0.05. The protein was eluted with 200 ml of 3mM folate buffer (10mM KH_2PO_4, 0.1mM EDTA, 1M KCl, 1mM folate, pH 9) at 1ml/min, collected in 8ml fractions. The gradient program then increased the elution buffer to 15mM folate over the next 100 minutes, and finished with a further 200ml of 15mM folate buffer. The high DHFR activity eluted fractions were combined, and dialyzed first against dialysis buffer (50mM Tris, 1mM EDTA, 1M NaCl, pH 6) and then equilibration buffer (10mM Tris, 1mM EDTA, pH 7.2). Proteins anti-CD3-DHFR[2] were concentrated with a Millipore Amicon Ultrafiltration YM-30 Membrane to approximately 1mg/ml.

Size exclusion chromatography

After incubation at room temperature with 1.1 equivalent dimerizer MTX_2-C_9 in P500 buffer (0.5M NaCl, 50mM KH_2PO_4, 1mM EDTA, pH 7) with 5% (v/v) glycerol for 1 hour, the protein solution was injected into a Superdex G200 size exclusion column (Amersham Biosciences, USA), eluted with P500 buffer and the relative peak sizes quantitated by absorbance at 280nm.

***In vitro* cytotoxicity blocking assay**

HPB-MLT T leukemia cells (10^5) were plated in a 96-well flat-bottom plate in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2mM L-glutamine, 100ug/ml penicillin, and 100ug/ml streptomycin. Immunotoxin

($DTUM_3$) in varying concentrations was added to triplicate wells containing cells. The plates were incubated at 37 ºC, 10% CO_2 for 72 hours, and 20ul MTS solution (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega) was added per well for the last 4 hours of incubation. The absorbance at 490nm was measured to determine the cell proliferation.

***In vitro* competitive binding assay**

After pre-mixing a dilution series of each purified anti-CD3-$DHFR^2$ monomer, dimer with the subsaturating concentration of FITC-labeled UCHT-1 mAb for 10 minutes, 10^6 cells were incubated with antibody mixture in PBS supplemented with 1% BSA and 0.1% sodium azide for 30 minutes at room temperature. The cells were then washed and fluorescence intensities of cell bound FITC-labeled antibodies were quantitated by FACSCalibur flow cytometer. % inhibition of binding was calculated by subtracting the mean fluorescence observed at given competitor concentration from 100% binding, which was defined as mean fluorescence observed in the absence of competition, then divided by 100% binding. Relative affinities were calculated from the corresponding IC_{50} values according to the equation: $K_{D(I)}=IC_{50}/(1+[\text{FITC-UCHT1}]/K_{D(\text{FITC-UCHT1})})$, where I is the unlabeled inhibitor, [FITC-UCHT1] is the concentration of FITC-UCHT1 used in the competitive reaction, $K_{D(\text{FITC-UCHT1})}$ is the binding affinity of FITC-UCHT1, IC_{50} is the concentration of the inhibitor that yields 50% inhibition of binding. $K_{D(\text{FITC-UCHT1})}$ was determined as previously described by fitting mean fluorescences of cell bound FITC-labeled UCHT-1 observed at different concentrations

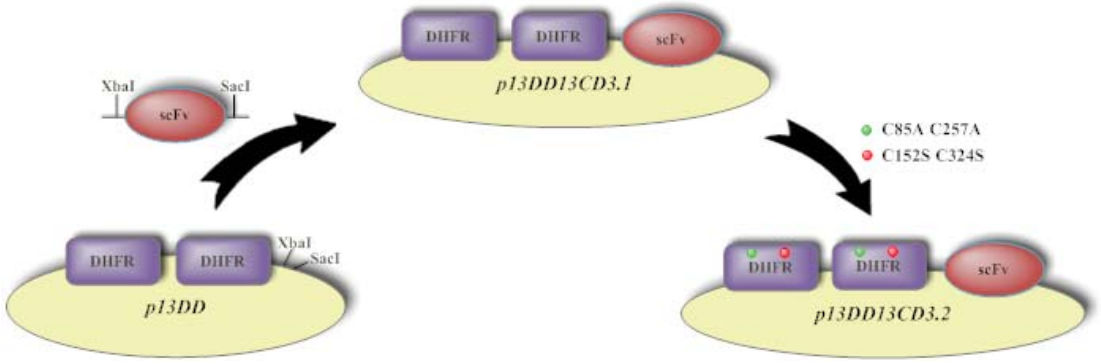
of FITC-labeled UCHT-1 to the Lineweaver-Burk equation: $1/F=1/F_{max}+(K_D/F_{max})(1/[FITC\text{-}UCHT1])$.

Competitive disassembly of anti-CD3-DHFR^2 nanorings

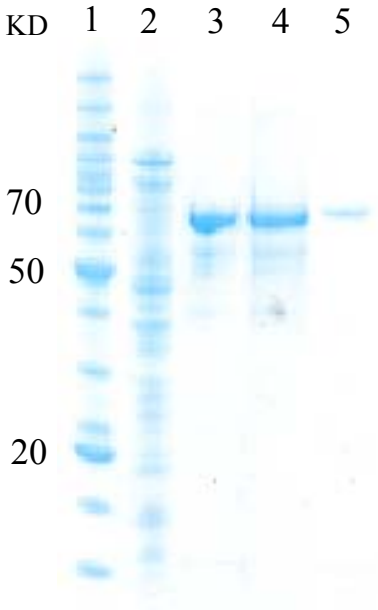
Competitive Disassembly experiments were performed by preparing anti-CD3-DHFR^2 dimer in P500 buffer mixed with excess amount of trimethoprim, which were then incubated for 20 hours at room temperature. Disassembly of anti-CD3-DHFR^2 dimer was verified via Size Exclusion Chromatography.

Confocal microscopy

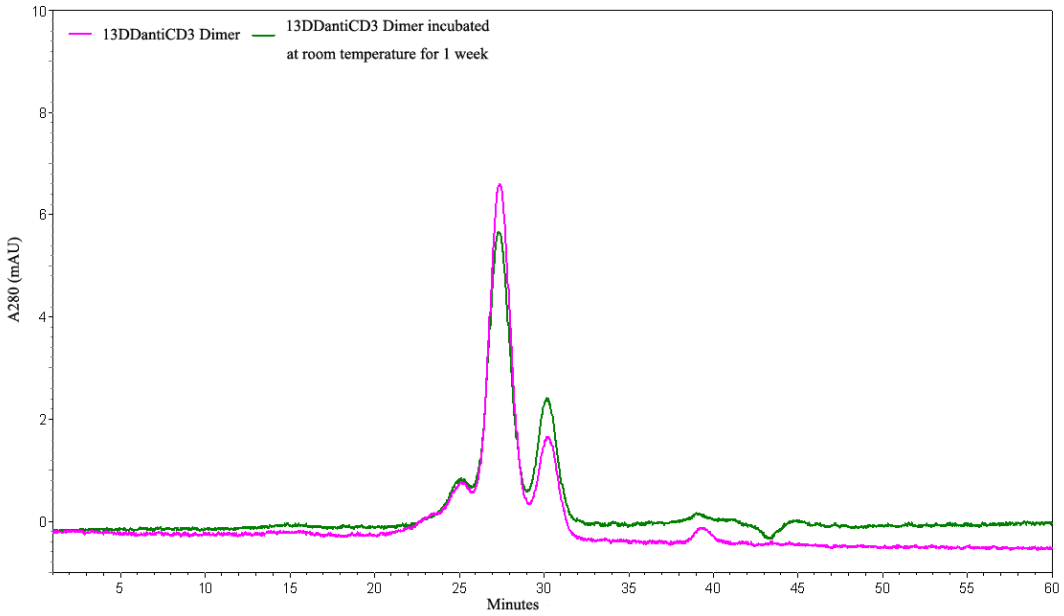
Anti-CD3-DHFR^2 monomer and dimer were firstly FITC labeled by using EZ-Label^{TM} FITC protein labeling kit. HPB-MLT T leukemia cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2mM L-glutamine, 100ug/ml penicillin, and 100ug/ml streptomycin at 37 ºC in 10% CO_2 atomosphere. Cells ($5x10^5$) were incubated with FITC-labeled anti-CD3-DHFR^2 monomer, or dimer in PBS at 37 ºC, in 10% CO_2 for 30 min on Poly Prep Slides^{TM} coated with poly-L-lysine (Sigma). They were then washed twice in PBS. The cells on the slides were fixed for 10 min in 2% paraformaldehyde in PBS, followed by staining cell membranes with 0.0005% concanavalin A-Alexa Fluor 594 solution (Molecular Probes) for 30 min. After washing twice in PBS, cells were embedded in prolong gold antifade mounting medium (Invitrogen). Pictures were taken within inner sections of the cells by sequential scanning using a confocal microscope.



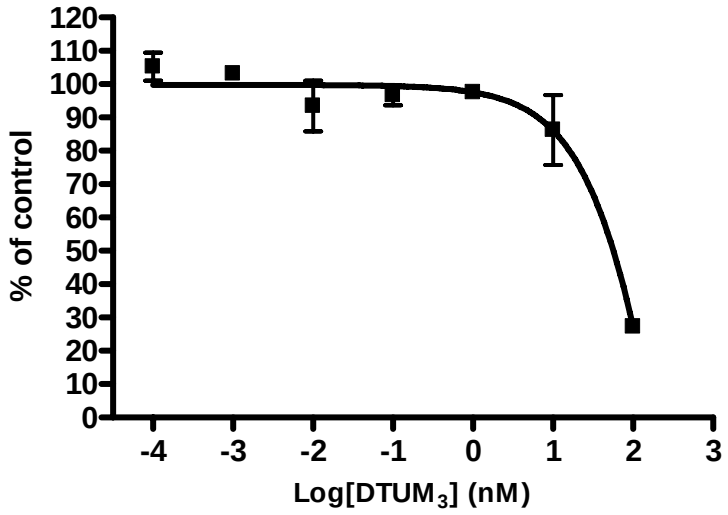
Supplementary Figure 1. Scheme for the plasmid construction.



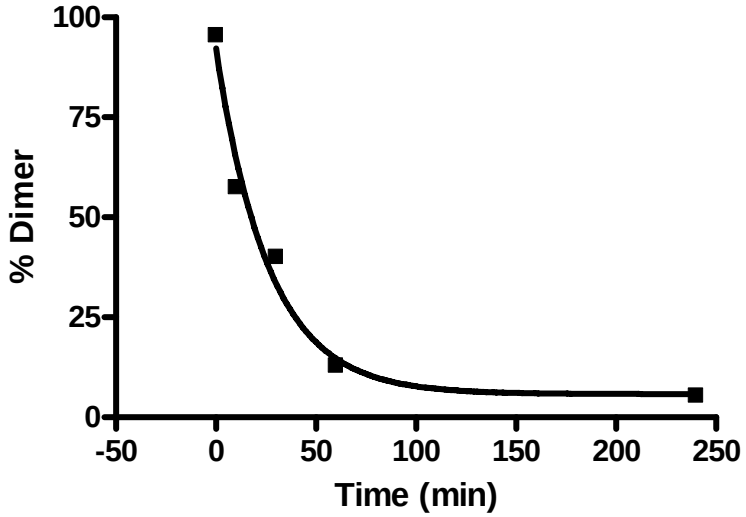
Supplementary Figure 2. SDS-PAGE analysis of anti-CD3-DHFR^2 protein expression. Lane 1: marker; Lane 2: cells without IPTG induction; Lane 3: overexpressed protein in inclusion body; Lane 4: unfolded protein; Lane 5: pure refolded protein.



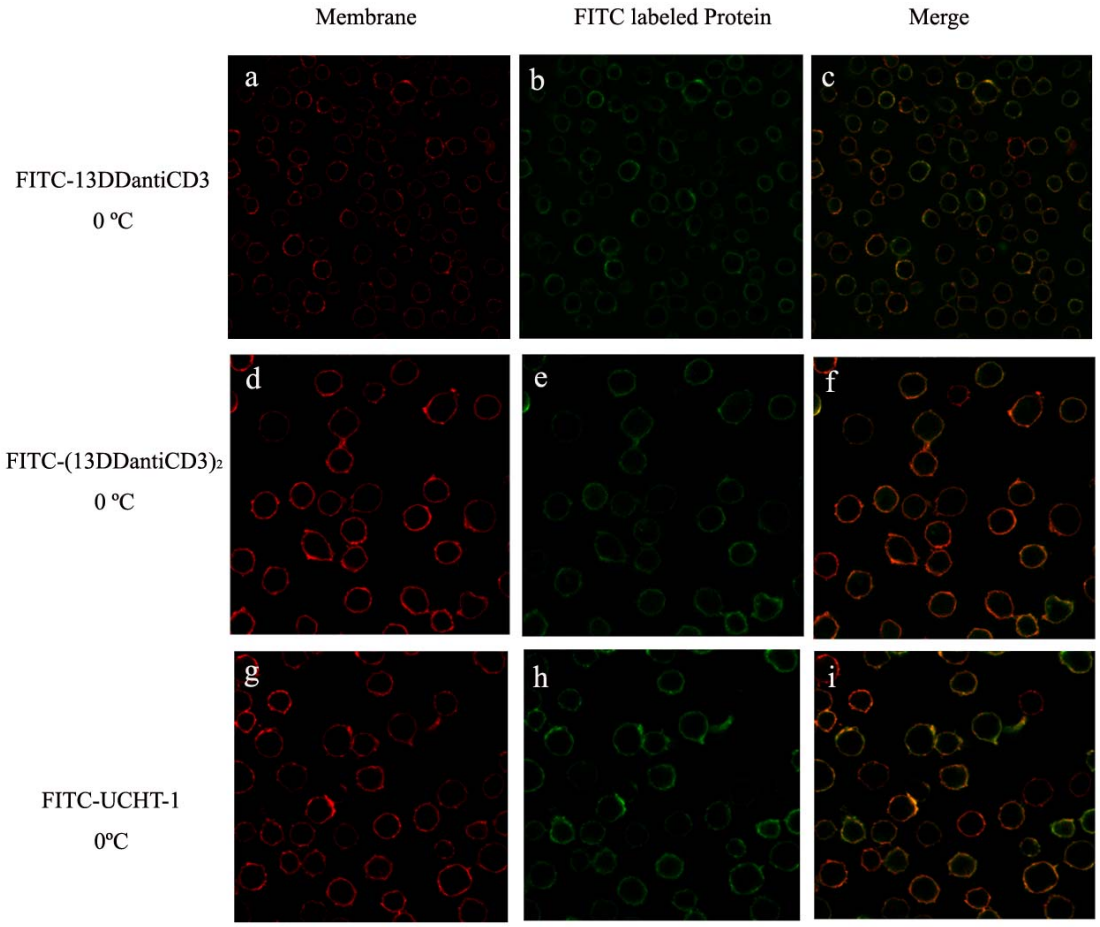
Supplementary Figure 3. Stability of anti-CD3-DHFR^2 dimer was characterized with size exclusion chromatography. Red curve showed anti-CD3-DHFR^2 dimer right after separated from dimerization reaction, while green curve represented anti-CD3-DHFR^2 dimer after incubating at room temperature for 1 week.



Supplementary Figure 4. Binding affinity of UCHT-1 to HPB-MLT T leukemia cells was characterized by i*n vitro* cytotoxicity blocking assay. Plot displayed cytotoxicity of immunotoxin DTUM3 blocked by 100nM UCHT-1.



Supplementary Figure 5. Time course study of disassembly of anti-CD3-DHFR^2 dimer by incubation with excess amount of trimethoprim. %Dimer: Percentage of dimer left in the disassembly reaction after incubating with trimethoprim at different time points.



Supplementary Figure 6. Confocal images of intracellular distribution of FITC-labeled anti-CD3-DHFR^2 monomer, dimer or UCHT-1 at 0ºC. HPB-MLT cells were incubated with FITC-labeled anti-CD3-DHFR^2 monomer (a-c),

FITC-labeled anti-CD3-DHFR^2 dimer (d-f), or FITC-labeled UCHT-1 (g-i) for 2 hours at 0ºC. Cell membranes were stained with concanavalin A-Alexa Fluor 594 (Red). Superposition of red channel and green channel were shown in images c, f, and i.