Protein Macrocyclization

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Self-Assembly of Antibodies by Chemical Induction**

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

The ability to clone and express the variable (V_H and V_L) and constant (C_H and C_L) domains of monoclonal antibodies has made the construction of genetically engineered antibodies possible.[1–3] Antibody fragments, such as Fabs (antigen-binding regions), have been fused to self-assembling protein domains, such as leucine zipper regions, of the transcription factors or to the C_L and C_{H1} domains of immunoglobulin G (IgG).[4,5] Recombinant antibodies, referred to as single-chain antibodies (scFvs), have been constructed by fusing the V_H and V_L domains of an antibody variable region (Fv) through a peptide linker. This advance resulted in the development of a variety of approaches for the preparation of the corresponding fusion relatives, diabodies (bivalent) and tandabs (tetravalent), which consist of a single polypeptide.[6–10] Although the current methods of preparing recombinant antibodies have a variety of advantages, in general, control over their assembly and disassembly is not possible without resorting to conditions for protein unfolding. Since the molecular weight and dimensions of engineered antibodies have a significant influence on their in vivo avidity, biodistribution, and pharmacokinetics, a method that enables these parameters to be altered temporally by chemically controlled assembly and disassembly would in principle enhance the therapeutic and diagnostic utility of recombinant antibodies.[11,12]

Recently, we developed a protocol for the preparation of self-assembling protein macrocyclic oligomers, or nanorings, of dihydrofolate reductase fusion proteins ($DHFR^2$) by chemical induction with bismethotrexate (bis-MTX). Self-assembling nanorings ranging in diameter from 7 to 30 nm could be prepared readily from two to eight $DHFR^2$ monomers.[13] The nanoring size was found to be governed by the specific length and composition of the peptide inserted between the DHFRs. For example, when the linker peptide between the DHFR molecules was a flexible linker containing 13 amino acid residues, dimer formation was observed almost exclusively upon incubation with bis-MTX. In comparison, octamers were observed when the peptide between the DHFR molecules was a single glycine residue. Inspection of the X-ray crystal structure of the DHFR dimer formed with bis-MTX suggested that proteins, such as scFvs, could be appended to $DHFR^2$ fusion proteins without loss of their ability to undergo chemically induced macrocyclic oligomerization.[14] Thus, we hypothesized that scFv–$DHFR^2$ fusion proteins could be induced by bis-MTX to self-assemble into bivalent, tetravalent, and octavalent antibody nanorings. To test our hypothesis, we prepared a $DHFR^2$–scFv fusion protein that binds to the T-cell antigen CD3 epsilon of the human T-cell receptor. The binding affinities and cell-surface behavior of the monovalent and bivalent scFv fusion protein were compared with those for the parental monoclonal antibody. We also assessed the ability of trimethoprim, a nontoxic competitive inhibitor of DHFR and an antibiotic approved by the US Food and Drug Administration (FDA), to carry out disassembly of the bivalent antibody nanoring (Figure 1 a).

To prepare the DHFR–DHFR–anti-CD3 scFv fusion protein, we first prepared the $DHFR^2$ expression plasmid p13DD13CD3.1 by ligating the short double-digested expression plasmid with the PCR product excised from Mo3.pGEMT.e that encodes anti-CD3 scFv (see Figure 1 in the Supporting Information). BL21 cells were transformed with p13DD13CD3.1. A substantial amount of insoluble protein (inclusion bodies) was observed, but no soluble protein (see Figure 2 in the Supporting Information). We employed the method developed previously by Vallera et al.[15] for the preparation of bacterially expressed scFvs by oxidation with air in the presence of sodium *N*-lauroylsarcosine (SLS); however, DHFR activity was not detectable, which indicated that the fusion protein was folded incorrectly and unlikely to bind bis-MTX.[16] Recognizing that each DHFR molecule contained two cysteine residues and that the oxidative conditions necessary for formation of the disulfide bond within the scFv may result in inappropriate disulfide-bond formation within a DHFR molecule, we prepared the quadruple $DHFR^2$ cysteine mutant C85A/C152S/C257A/C324S, as it had been shown previously that neither the DHFR mutant C85A nor C152S resulted in loss of DHFR activity.[17] Upon solubilization and refolding by oxidation in air with sodium *N*-lauroylsarcosine, the quadruple mutant was found to have the same specific DHFR activity as wild-type DHFR and could be purified readily by methotrexate-affinity chromatography.[13]

The purified antibody fusion protein, anti-CD3–$DHFR^2$, was incubated for 1 hour with bis-MTX (1 equiv), and the product was analyzed by size-exclusion chromatography

[*] Dr. C. R. Wagner
Department of Medicinal Chemistry, University of Minnesota
8-101 Weaver-Densford Hall, 308 Harvard Street
Minneapolis, MN 55455 (USA)
Fax: (+1) 612-624-0139
E-mail: wagne003@tc.umn.edu
Q. Li
Department of Chemistry, University of Minnesota
Minneapolis, MN 55455 (USA)
D. Hapka
College of Pharmacy, University of Minnesota
Minneapolis, MN 55455 (USA)
Dr. H. Chen, Dr. D. A. Vallera
Department of Therapeutic Radiology, University of Minnesota
Minneapolis, MN 55455 (USA)

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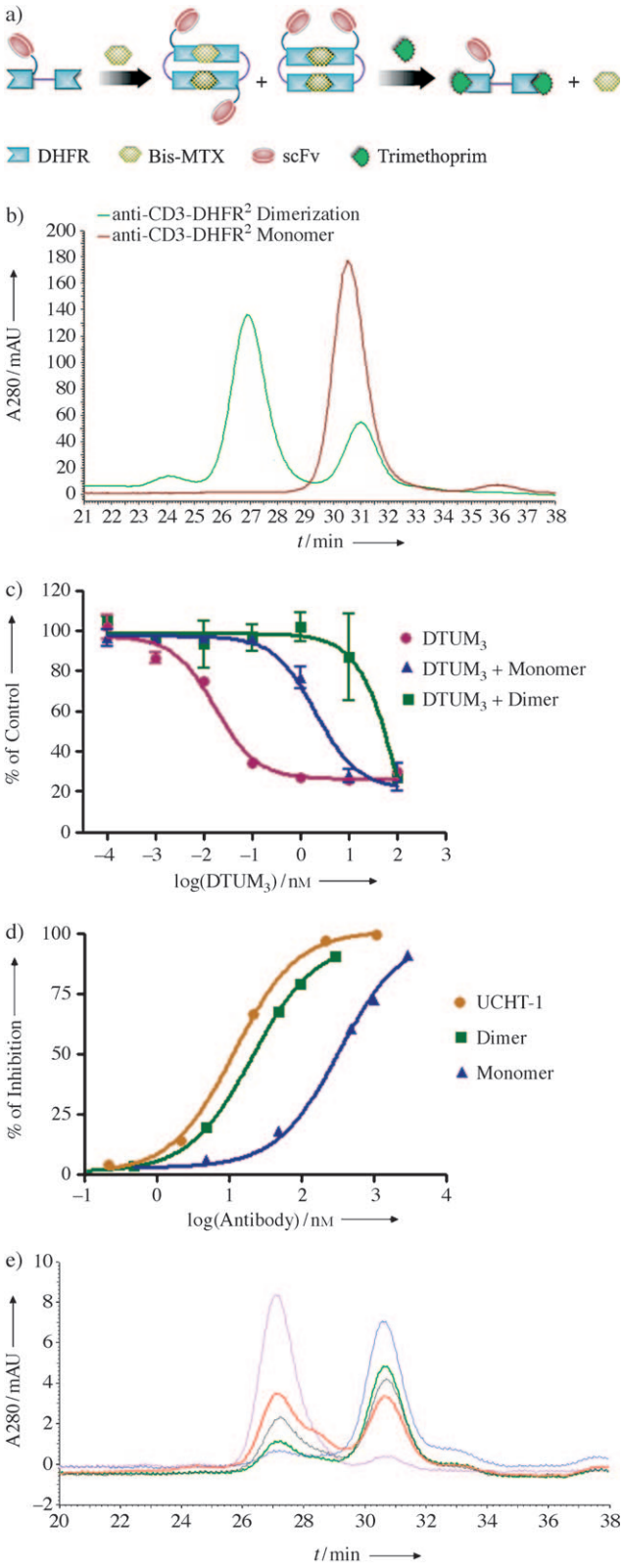

Figure 1. Design and evolution of bivalent single-chain antibodies. a) Assembly and disassembly of bivalent single-chain antibodies. b) The assembly of anti-CD3–DHFR^2 dimer was characterized by size-exclusion chromatography. Red curve: anti-CD3–DHFR^2 monomer; green curve: induced dimerization of anti-CD3–DHFR^2 with bis-MTX. c) The binding affinities of anti-CD3–DHFR^2 monomer and dimer for HPB-MLT T leukemia cells were compared in an in vitro cytotoxicity-blocking assay. Plot shows the cytotoxicity of the immunotoxin $DTUM_3$ alone (purple dots), $DTUM_3$ in the presence of anti-CD3–DHFR^2 monomer (100 nM, blue triangles), and $DTUM_3$ in the presence of anti-CD3–DHFR^2 dimer (100 nM, green squares). d) Flow cytometric competitive-binding assay to determine the disassociation constant of anti-CD3–DHFR^2 monomer (blue triangles), the dimer (green squares), and UCHT-1 (orange dots). e) The disassembly of anti-CD3–DHFR^2 dimer by trimethoprim was characterized by size-exclusion chromatography. Pink curve: anti-CD3–DHFR^2 dimer; red, black, green, and blue curves: disassembled anti-CD3–DHFR^2 after incubation with trimethoprim for 10, 30, 60, and 240 min, respectively.

(SEC; Figure 1b). Dimeric anti-CD3–DHFR^2 (138 kD, 69%) eluted from the superdex G200 column with a retention time of 27 min, whereas monomeric anti-CD3–DHFR^2 (68 kD) eluted with a retention time of 30.5 min. Small amounts of the corresponding trimer (24.4 min, 3%) and the monomer (28%) were observed. As observed previously for the oligomerization of DHFR^2 fusion proteins containing a 13 amino acid linker, the larger species disappeared with time.[13] Furthermore, in analogy with the oligomerization of DHFR^2 containing a 13 amino acid linker, a small amount of remaining monomeric anti-CD3–DHFR^2 (25%) was observable, even when 5 equivalents of bis-MTX were used.[13] Given the slightly increased retention time of this species (31 min) with respect to that of the monomer in the absence of bis-MTX (30.5 min) and the observation that the amount of remaining monomer is considerably lower at high monomer concentrations, it is highly probable that this species results from the intramolecular macrocyclization of anti-CD3–DHFR^2. Peak broadening was observed for the anti-CD3–DHFR^2 dimer relative to DHFR^2 and is expected given the possibility of the formation of *trans* and *cis* isomers with respect to the position of the appended scFvs. Future strategies for DHFR heterodimerization should enable the exclusive preparation of either the *cis* or the *trans* isomer of the dimer. The stability of the self-assembled antibodies was assessed by incubating dimerized anti-CD3–DHFR^2 at room temperature for 1 week, followed by reanalysis by SEC. No appreciable change in the species distribution or amount of dimeric self-assembled antibody was observed (see Figure 3 in the Supporting Information).

To determine the binding affinity of anti-CD3–DHFR^2 for CD3, we first compared the ability of the anti-CD3–DHFR^2 monomer and dimer to protect CD3+ human T-leukemia HPB-MLT cells upon simultaneous treatment with diphtheria anti-CD3 immunotoxin[18] ($DTUM_3$, $IC_{50} = 0.0337 \pm 0.0116$ nM; Figure 1c). The anti-CD3 scFv was highly active and increased the IC_{50} value of the immunotoxin more than 100-fold ($IC_{50} = 3.48 \pm 1.33$ nM). Protection from the immunotoxin was enhanced further by a factor of 16 ($IC_{50} = 55.7 \pm 13.6$ nM) when the cells were incubated with dimeric anti-CD3–DHFR^2. Thus, the IC_{50} value was increased approximately 1600-fold in total. The parental antibody, UCHT-1 ($IC_{50} = 56.6 \pm 2.9$ nM), was found to provide the same degree of protection as dimerized anti-CD3–DHFR^2. Thus, dimeric anti-CD3–DHFR^2 is a fully functioning antibody mimic (see Figure 4 in the Supporting Information).

To determine quantitatively the binding affinity of the chemically self-assembled diabody, the dissociation constants (K_d) for monomeric and dimeric anti-CD3–DHFR^2 and UCHT-1 were evaluated in a flow cytometric competitive-binding assay (Figure 1 d). The K_d value of 3.5 ± 0.2 nM found for dimeric anti-CD3–DHFR^2 is comparable to that of the parental monoclonal antibody, UCHT1 ($K_d = 1.8 \pm 0.2$ nM). Clearly, the bivalent antibodies have superior affinities to that of the monovalent scFv fusion protein, as the K_d value for monomeric anti-CD3–DHFR^2 ($K_d = 51 \pm 9.8$ nM) was found to be approximately 16-fold lower than the value for dimeric anti-CD3–DHFR^2. Taken together, the results from the cellular-protection and cell-binding studies demonstrate that the self-assembled diabody is nearly identical in affinity to the parental monoclonal antibody.

One of the distinct advantages of a chemically induced self-assembly approach dependent on a bivalent ligand over other methods for the preparation of recombinant antibodies is the potential to temporally initiate disassembly by the addition of a competitive monomeric ligand. Since trimethoprim is an FDA-approved antibiotic that, like bis-MTX, binds tightly to the DHFR active site, we investigated its ability to initiate DHFR-oligomer disassembly by incubating dimeric anti-CD3–DHFR^2 with a 10-fold excess of trimethoprim, followed by determination of the amount of dimer and monomer present at various time points. As can be seen in Figure 1 e, disassembly was initiated rapidly with 40 % conversion of the dimeric protein into the monomer within 10 min and approximately 100 % conversion within 1 h. The half-life for disassembly ($t_{1/2}$) at a 10 uM concentration of trimethoprim was found to be 18 min, which corresponds to a rate of 0.0385 min^{-1} (see Figure 5 in the Supporting Information). Therefore, despite the lower affinity of trimethoprim for DHFR[19] ($K_d = 4.6$ nM) relative to that of bis-MTX[14] ($K_d = 21$ pM), rapid disassembly of the self-assembled antibodies is possible. The conversion of the cyclic oligomer into less-stable intermediate linear species undoubtedly contributes to the observed rapid disassembly.

Confocal laser scanning microscopy was used to characterize the interaction of fluorescently labeled monomeric and dimeric anti-CD3–DHFR^2 and UCHT-1 with living cells (Figure 2). Upon incubation at 37 °C, monomeric and dimeric anti-CD3–DHFR^2 were found to localize on the surface of CD3+ HPB-MLT cells, followed by intracellular uptake. Colocalization studies with a lysosomal specific red fluorescent dye, LysoTracker Red DND-99, identified these compartments as late endosomes (Figure 3). Consistent with a mechanism relying on receptor and therefore energy-dependent endocytosis, uptake of the recombinant antibodies was blocked by incubation at 4 °C (see Figure 6 in the Supporting Information). No observable difference in the intracellular location of the recombinant antibodies and UCHT-1 was detectable. Thus, both monomeric and dimeric anti-CD3–DHFR^2 and the parental monoclonal antibody appear to interact with cells in a similar manner.

In conclusion, we have developed a modular method for the reversible preparation of stable recombinant antibodies by chemically induced self-assembly. Unlike previous methods for the preparation of recombinant antibodies, our approach enables the oligomeric state of the antibody to be temporally controlled by a FDA-approved drug. This feature is particularly important for the control not only of antibody avidity, but also of molecular weight, a key determinant of the in vivo pharmacokinetics of antibodies. Since we have demonstrated previously that DHFR^2 fusion proteins can be self-assembled into protein nanorings comprised of two to eight subunits, we envision that our approach will enable the preparation of antibody nanorings that incorporate between two and eight scFvs. Our unique approach will then enable

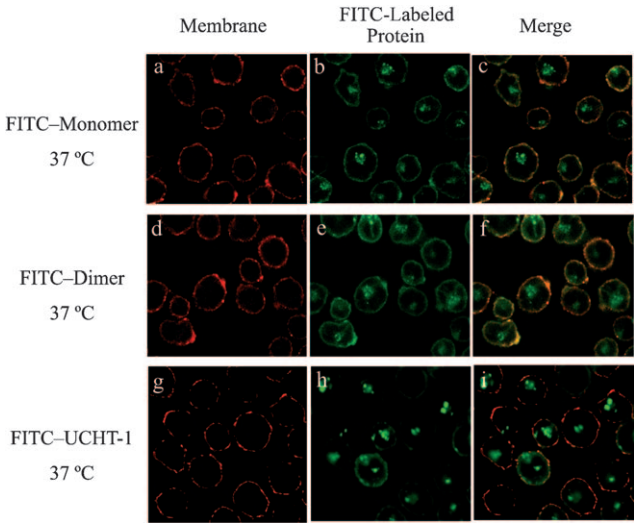

Figure 2. Confocal images of the intracellular distribution of FITC-labeled anti-CD3–DHFR^2 monomer, anti-CD3–DHFR^2 dimer, and UCHT-1 at 37 °C. HPB-MLT cells were incubated with FITC-labeled anti-CD3–DHFR^2 monomer (a–c), FITC-labeled anti-CD3–DHFR^2 dimer (d–f), or FITC-labeled UCHT-1 (g–i) for 2 h at 37 °C. The cell membranes were stained with concanavalin A–Alexa Fluor 594 (red). Superposition of the red and green channels is shown in images c, f, and i. FITC = fluorescein isothiocyanate.

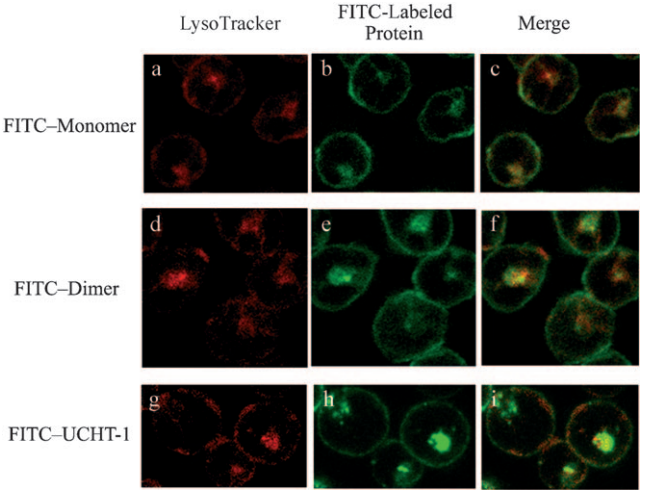

Figure 3. Confocal images of HPB-MLT T leukemia cells incubated with FITC-labeled anti-CD3–DHFR^2 monomer (b), anti-CD3–DHFR^2 dimer (e), and UCHT-1 (h) for 30 min at 37 °C. Images a, d, and g show lysosomal compartments of the cells. Superposition of the red and green channels is shown in images c, f, and i.

the disassembly of these nanorings into subunits with a common molecular weight of approximately 60 kDa for rapid elimination.[2,20] Furthermore, we are preparing trifunctional bis-MTX analogues that are capable of delivering fluorophores, radionuclides, and drugs to scFv-targeted tissues. The results of these studies will be reported in due course.

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