Self-Assembling Antibodies by Chemical Induction**

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The ability to clone and express the variable (V_H & V_L) and constant (C_H & C_L) domains of monoclonal antibodies has made the construction of genetically engineered antibodies possible.[1-3] Antibody fragments, such as Fabs, 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 IgG.[4, 5] Recombinant antibodies, referred to as single chain antibodies (scFv's), have been constructed by fusing the V_H and V_L domains of an antibody variable region (Fv) via 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) consisting 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 necessary for protein unfolding. Since the molecular weight and dimensions of engineered antibodies is a significant determinant of their in vivo avidity, biodistribution and pharmacokinetics, a method allowing these parameters to be temporally altered by chemically controlled assembly and disassembly would in principle enhance the therapeutic and diagnostc utility of recombinant antibodies[11, 12].

Recently, we have 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 bis-methotrexate (Bis-MTX). Self-assembling nanorings ranging in diameter from 7 to 30 nm could easily be prepared from two to eight $DHFR^2$ monomers, respectively.[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 $DHFR^2$ was a 13 amino acid flexible linker, dimer formation was

observed almost exclusively after incubation with Bis-MTX. In comparison, octamers were observed when the peptide between the $DHFR^2$ was a single glycine. Inspection of the x-ray crystal structure of the Bis-MTX dimerized DHFR suggested that proteins, such as scFv's, 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, a $DHFR^2$ scFv fusion protein that binds to the T-cell antigen, CD3 epsilon, of the human T-cell receptor was prepared. The binding affinities and cell surface behavior of the monovalent and bivalent scFv fusion protein were compared with those for the parental monoclonal antibody. (Figure 1a) In addition, we assessed the ability of trimethoprim, a non-toxic competitive inhibitor of DHFR and FDA approved antiobiotic, to carry out disassembly of the bivalent antibody nanoring. (Figure 1a)

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 and anti-CD3 scFv encoding PCR product excised from Mo3.pGEMT.e by PCR followed by ligation. (See Supplementary Fig. 1 online) BL21 cells were transformed with p13DD13CD3.1 and substantial amounts of insoluble (inclusion bodies), but not soluble protein was observed. (See Supplementary Fig. 2 online) The sodium N-lauroyl-sarcosine (SLS) air oxidation method previously developed by Vallera et al.[15] for the preparation of bacterially expressed scFv's was employed; however, DHFR activity was not detectable, indicating that the fusion protein was incorrectly folded and unlikely to bind Bis-MTX.[16] Recognizing that each DHFR contained two cysteines and that the oxidative conditions necessary for scFv disulfide bond formation may result in inappropriate DHFR internal disulfide formation. we prepared the quadruple $DHFR^2$ cysteine mutant (C85A C152S C257A C324S), since it had been previously shown that neither the DHFR mutant, C85A nor C152S resulted in loss of DHFR activity,[17] Upon solubilization and refolding by the sodium N-lauroyl-sarcosine (SLS) air oxidation method, the quadruple mutant was found to be soluble with the same specific DHFR activity as wild type DHFR (Data not shown), and easily purified by methotrexate affinity chromatography.[13]

The purified antibody fusion protein, anti-CD3-$DHFR^2$ was incubated for 1 hr with one equivalent of Bis-MTX and analyzed by size exclusion chromatography (SEC). (Figure 1b) Dimeric anti-CD3-$DHFR^2$ (138KD, 69%) was observed to elute from the Superdex G200 column at a retention time of 27 min, while monomeric anti-CD3-$DHFR^2$ (68KD) eluted with a retention time of 30.5. A small amount of trimer (24.4 min, 3%) and monomer (28%) was 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] In addition, similar to $DHFR^2$ containing a 13 amino acid linker, a small amount of remaining monomeric anti-CD3-$DHFR^2$ (25%) was observable, even when incubated with as high as 5 equivalents of Bis-MTX.[13] (Data not shown) Given the slightly increased retention time (31 min vs. 30.5 min) relative to monomer not incubated with Bis-MTX 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 intramolecular macrocyclization of anti-CD3-$DHFR^2$. Relative to $DHFR^2$, peak broadening of the anti-CD3-$DHFR^2$ dimer was observed and is expected given the possibility of trans and cis isomer formation with respect to the position of the appended scFv's. The future development of strategies for DHFR heterodimerization should allow the preparation of exclusively either the cis or trans dimer isomer to be obtained. The stability of the self-assembled antibodies was assessed by incubating dimerized anti-CD3-$DHFR^2$ at room temperature for 1 week followed by re-analysis by SEC. No appreciable change in the

species distribution or amount of dimeric self-assembled antibody was observed. (See Supplementary Fig. 3 online)

To determine the binding affinity of the of anti-CD3-DHFR^2 to CD3, we first compared the ability of the anti-CD3-DHFR^2 monomer and dimer to protect CD3+ human T-leukemia HPB-MLT cells from the simultaneous treatment with the immunotoxin, diphtheria-anti-CD3 immunotoxin[18] (DTUM$_3$, IC_{50} = 0.0337 ± 0.0116 nM). (Figure 1c) The anti-CD3 scFv was shown to be highly active by increasing the IC_{50} by greater than 100- fold (IC_{50} = 3.48 ± 1.33 nM). Protection from the immunotoxin was further enhanced by an additional 16- fold (IC_{50} = 55.7 ± 13.6 nM) when the cells were incubated with dimeric anti-CD3-DHFR^2, thus affording and overall protection of approximately 1600- fold. Consistent with a fully functioning antibody mimic, the parental antibody, UCHT-1 (IC_{50} = 56.6 ± 2.9 nM), was found to provide the same degree of protection as dimerized anti-CD3-DHFR^2. (See Supplementary Fig. 4 online)

To quantitatively determine the binding affinity of the chemically self-assembled diabody, the dissociation constants (K_d) for the monomic and dimeric anti-CD3-DHFR^2 and UCHT-1 were evaluated with a flow cytometric competitive binding assay. (Figure 1d) Compared to the parental monoclonal antibody, UCHT1 (K_d = 1.8 ± 0.2 nM), dimeric anti-CD3-DHFR^2 displayed a comparable K_d value of 3.5 ± 0.2 nM. Clearly, the bivalent antibodies have superior affinities over the monovalent scFv fusion protein, since the K_d value for monomeric anti-CD3-DHFR^2 (K_d = 51 ± 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.

Unlike other methods of preparing recombinant antibodies, one of the distinct advantages of a chemically induced self-assembly approach dependent on a bivalent ligand is the potential to temporally initiate disassembly by 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 1e, disassembly was rapidly initiated with 40% conversion of the dimeric protein to monomer occurring in 10 min and approximately 100% conversion within 1 hr. The half-life for disassembly ($t_{1/2}$) at 10 uM trimethoprim was found to be 18 min corresponding to a rate of 0.0385 min^{-1}, (See Supplementary Fig. 5 online) Therefore, despite the lower affinity of trimethoprim[19] (K_d = 4.6 nM) relative to Bis-MTX[14] (K_d = 21 pM), rapid disassembly of the self-assembled antibodies can be achieved. The conversion of the cyclic oligomer to 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) When incubated at 37°C, monomeric and dimeric anti-CD3-DHFR^2 were found to first localize on the surface of CD3+ HPB-MLT cells, followed by intracellular uptake. Co-localization studies with a lysosomal specific red fluoresent 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 Supplementary Fig. 6 online) No observable difference in the intracellular location of the recombinant antibodies and UCHT-1 was detectable. Consequently, both the monomeric and dimeric anti-CD3-DHFR^2 and the parental monoclonal 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 recombinant antibody methods, our approach allows the oligomeric state of the antibody to be temporally controlled by a FDA approved drug. This is particularly important for not only controlling antibody avidity, but also molecular weight, a key determinant of antibody in vivo pharmacokinetics. Since we have previously demonstrated DHFR[2] fusion proteins can be self-assembled into protein nanorings comprised of from two to eight subunits, we envision that our approach will allow preparation of antibody nanorings incorporating from two to eight scFv's. Our unique approach will then allow their disassembly into subunits with a common molecular weight of approximately 60 kDa, for rapid elimination.[2, 20] In addition, trifunctional Bis-MTX analogs are being prepared that are capable of delivering, fluorophores, radionuclides and drugs to scFv targeted tissues. The results of these studies will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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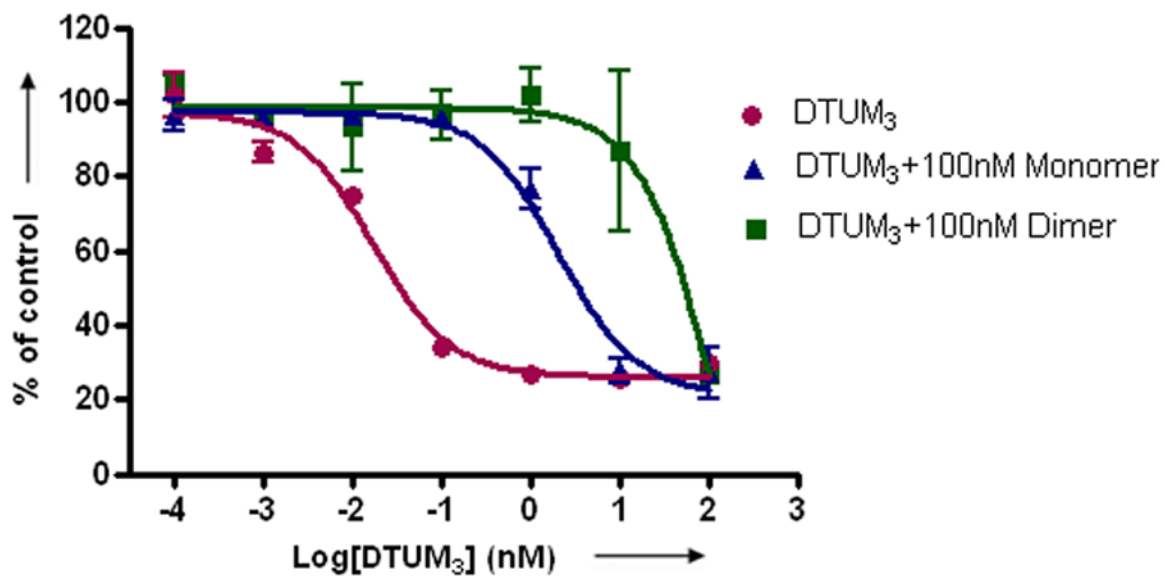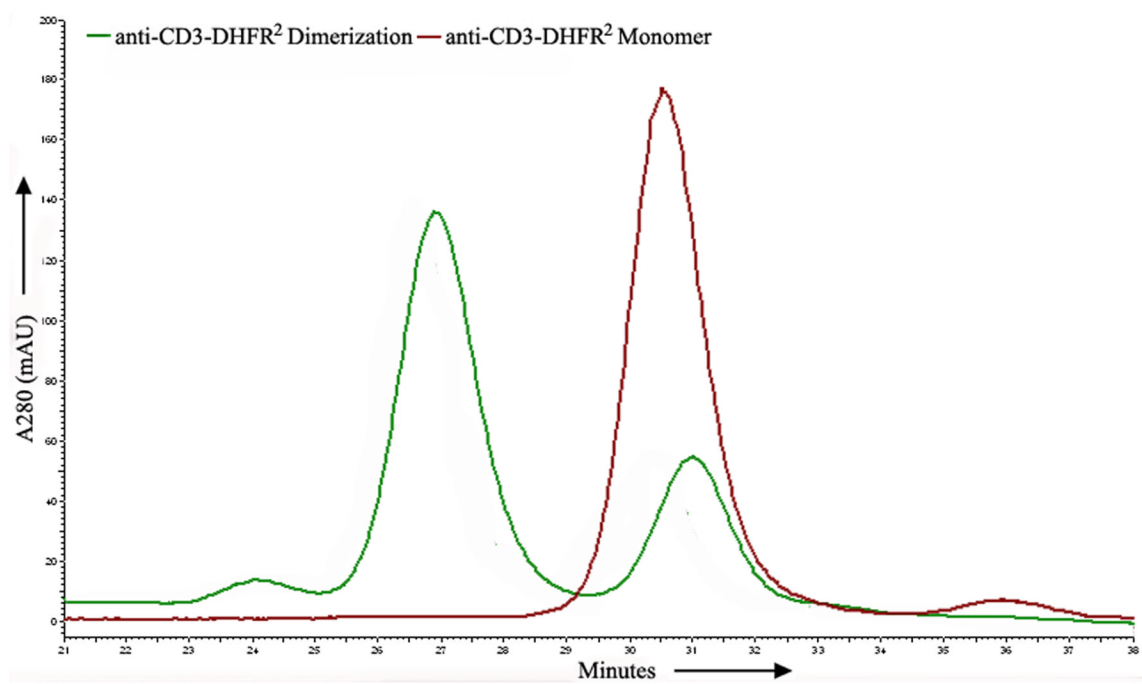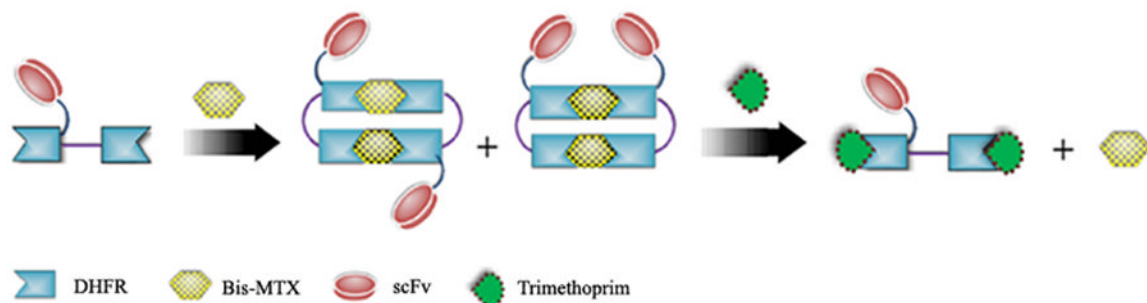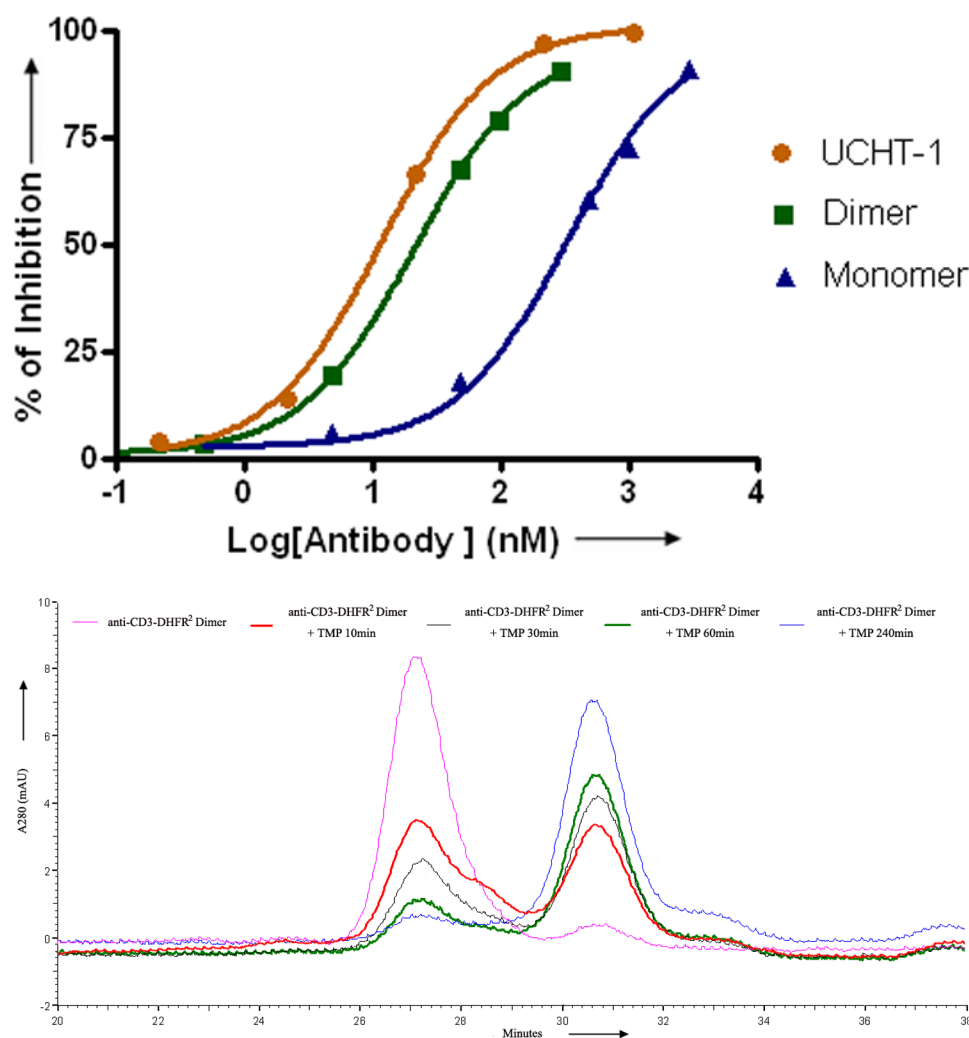
Figure 1. Design and evolution of bivalent single-chain antibody

a) Scheme for the assembly and disassembly of bivalent single-chain antibody. **b**) Assembly of anti-CD3-DHFR^2 dimer was characterized by size exclusion chromatography. Red curve showed anti-CD3-DHFR^2 monomer, while green curve represented the induced dimerization reaction of anti-CD3-DHFR^2 with Bis-MTX. **c**) Binding affinities of anti-CD3-DHFR^2 monomer and dimer to HPB-MLT T leukemia cells were compared by i*n vitro* cytotoxicity blocking assay. Plot displayed cytotoxicity of immunotoxin DTUM$_3$ alone (purple dots), cytotoxicity of DTUM3 blocked by 100nM anti-CD3-DHFR^2 monomer (blue triangles), and cytotoxicity of DTUM3 blocked by 100nM anti-CD3-DHFR^2 dimer (green squares). **d**) Flow cytometric competitive binding assay was used to determine disassociation constant of anti-CD3-DHFR^2 monomer (blue triangles), dimer (green squares) and UCHT-1 (orange dots). **e**) Disassembly of anti-CD3-DHFR^2 dimer by trimethoprim at different time points was characterized with size exclusion chromatography. Light purple curve showed anti-CD3-DHFR^2 dimer, while red, black, green, and blue curves displayed the disassembled anti-CD3-DHFR^2 after incubating with trimethoprim for 10, 30, 60, and 240 mins.
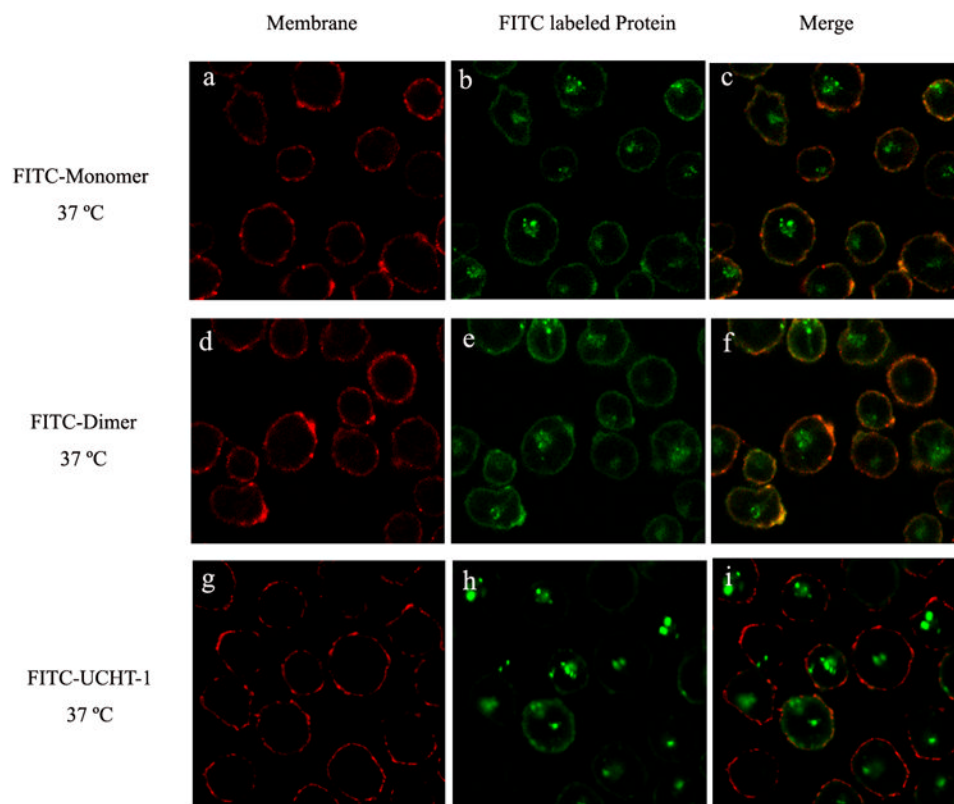
Figure 2.
Confocal images of intracellular distribution of FITC-labeled anti-CD3-DHFR^2 monomer, dimer or UCHT-1 at 37°C. HPB-MLT cells were incubated with FITC-labeled anti-CD3-DHFR^2 monomer (a-c), FITC-labeled anti-CD3-DHFR^2 dimer (d-f), or FITC-labeled UCHT-1 (g-i) for 2 hours at 37°C. Cell membranes were stained with concanavalin A-Alexa Fluor 594 (Red). Superposition of red channel and green channel were shown in images c, f, and i.
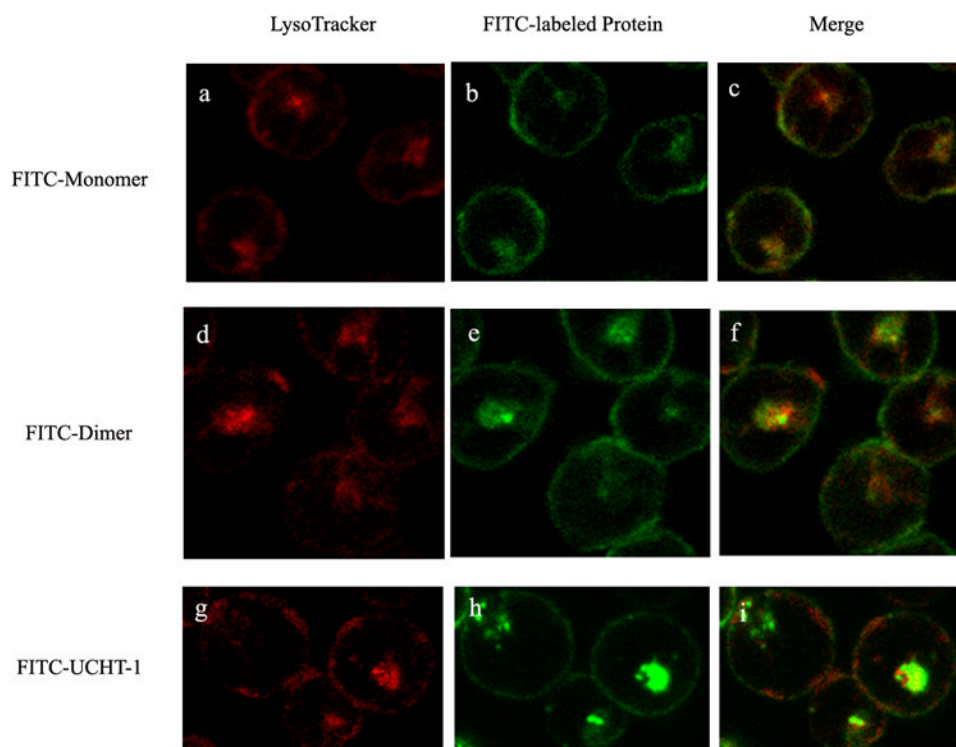
Figure 3.
Confocal images of HPB-MLT T leukemia cells incubated with FITC-labeled anti-CD3-DHFR^2 monomer (b) dimer (e) or UCHT-1 (h) for 30 mins at 37°C. Images a, d and g showed lysosomal compartments of the cells. Superposition of red channel and green channel were shown in images c, f, and i.