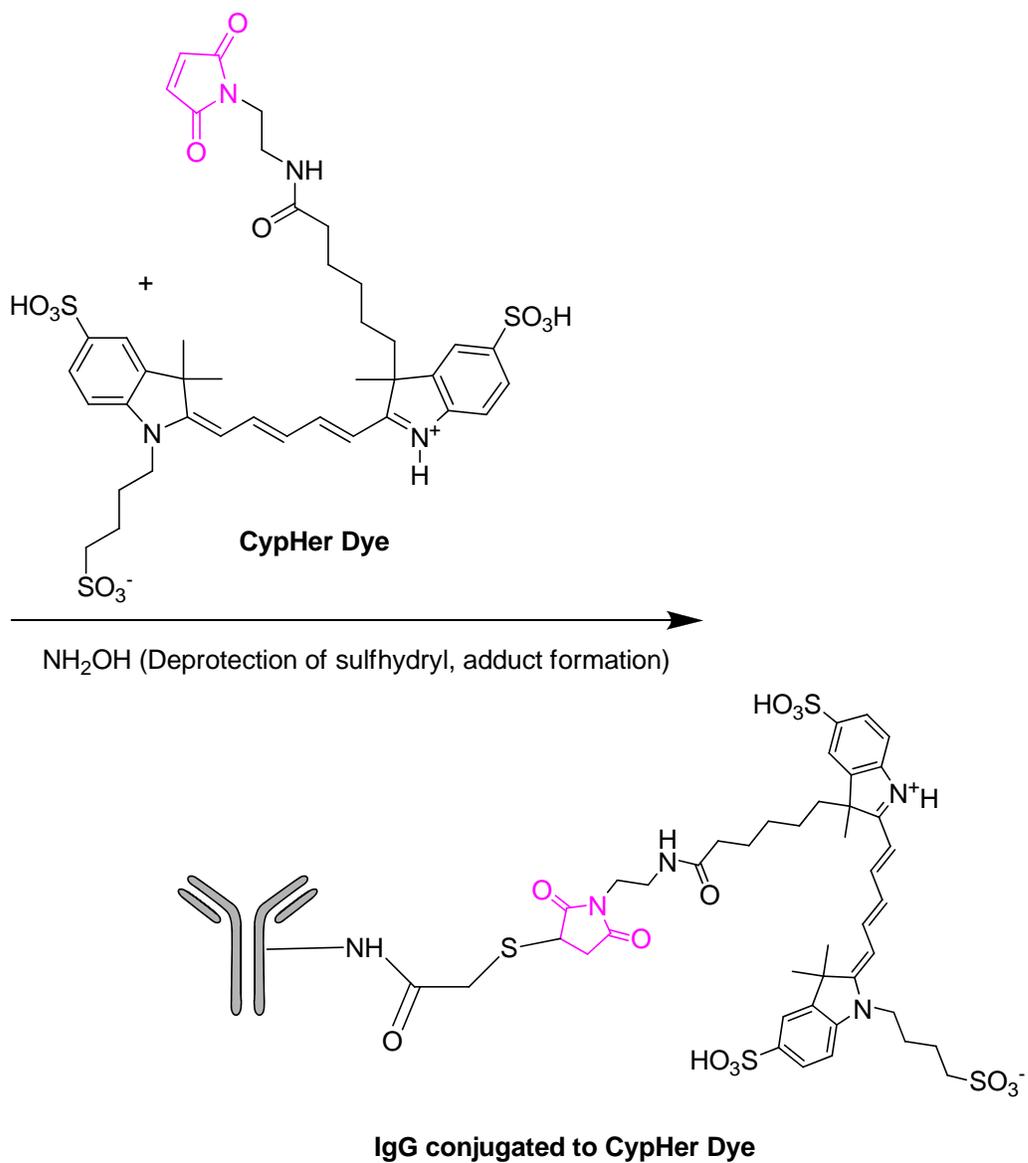
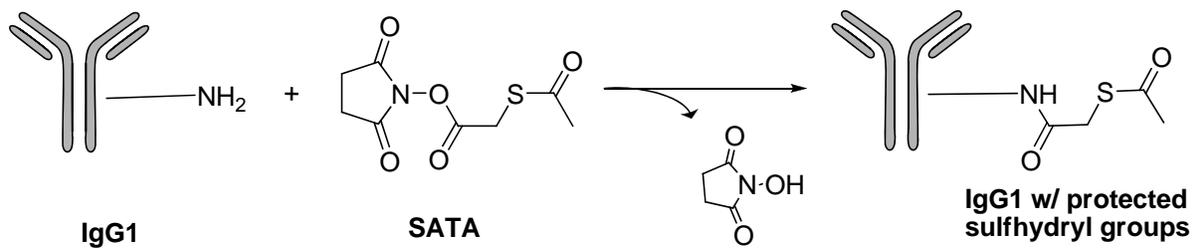


Scheme 2. Reaction scheme for the test-conjugation of the maleimido-Cypher dye conjugate to a representative thiol-containing protein.

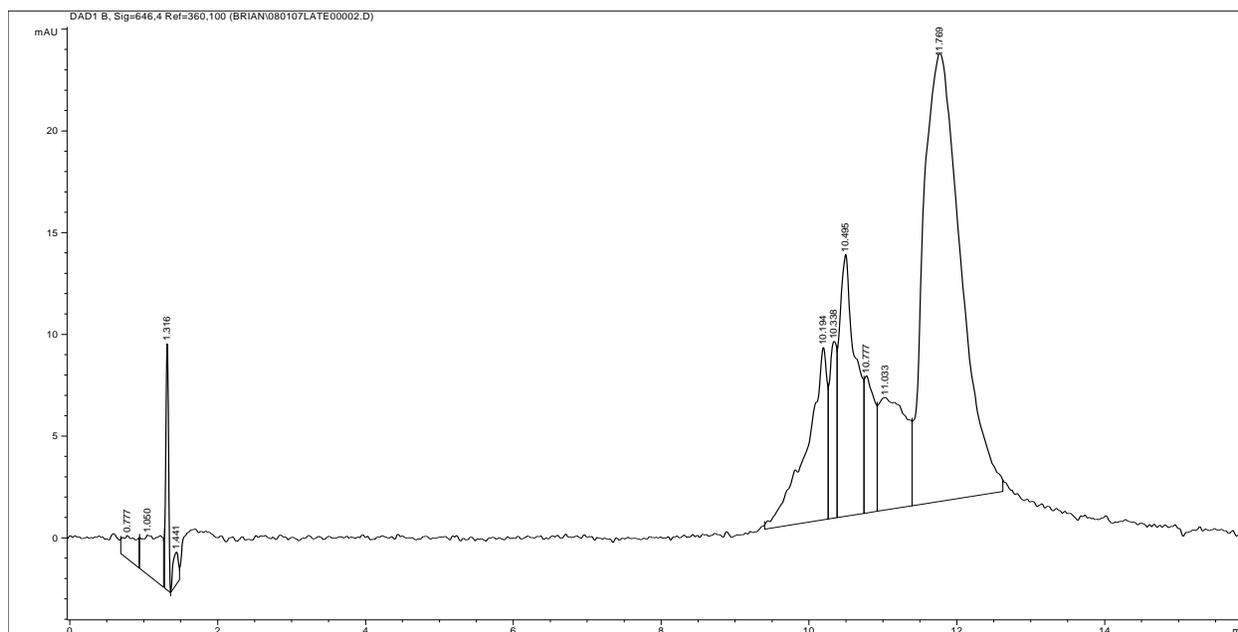


Experimental

General. HPLC was done on an Agilent 1200 System. Most reagents used are commercially available from multiple sources while the CypHer5E dye is available from Invitrogen.

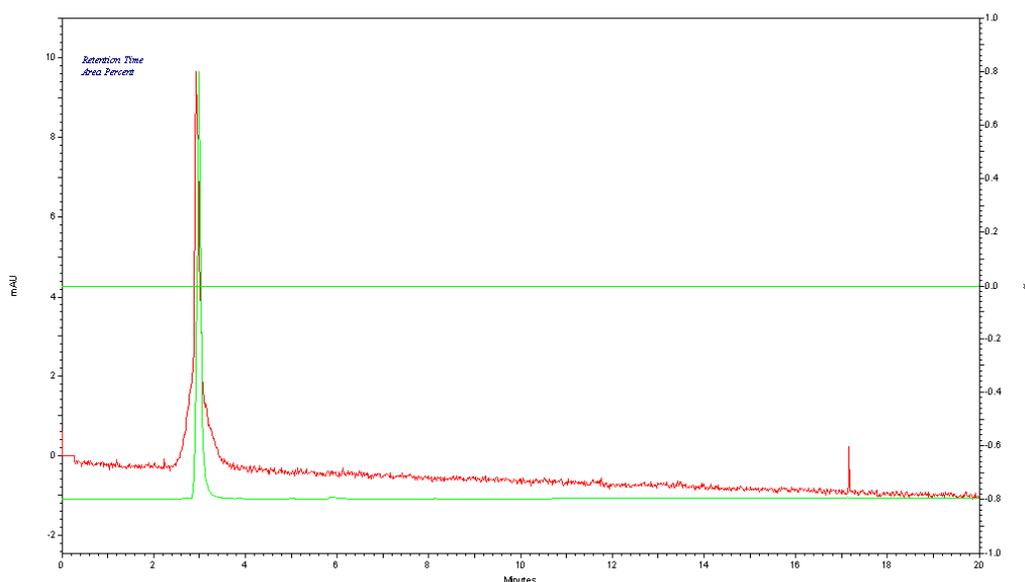
Maleimido CypHer5E Dye (2). CypHer5E (1) (2.0 mg, 2.4 μmol) and 1-(2-aminoethyl)maleimide (0.9 mg, 6.4 μmol) were dissolved in anhydrous DMF (100 μl) followed by the addition of DIPEA (0.9 μl , 5.1 μmol). The resulting solution was shaken in the dark for 24 hours at room temperature. It was purified by HPLC to give the conjugated product (2) as a red powder. The yield was determined to be 1.5 mg (73%). Analysis of the final product was performed using an Agilent 1200 Series HPLC System. Solvent A was 0.05% TFA in water and solvent B was 0.05% TFA in acetonitrile. An Agilent XDB-C₁₈, 3.5 μm , 3.0 x 150 mm column was used with a flow rate of 1.0 mL/minute. A linear gradient from 5-85% solvent B was used with the product (2) eluting at 11.8 minutes, $\lambda=646$ nm. The HPLC trace is shown in **Figure 1**.

Figure 1. HPLC data of Maleimido CypHer5E conjugate at absorbance 646 nm shows the product peak at retention time (11.8 min) with an estimated purity around 51%.



Bioconjugation of Maleimido-CypHer5E Dye to IgG₁. The IgG₁ antibody-CypHer5E dye conjugate was prepared according to **Scheme 1**. The antibody was placed in a solution of 50 mM sodium phosphate, pH 7.5, containing 10 mM EDTA. Protected sulfhydryl groups were added to the IgG by the addition of SATA (*N*-Succinimidyl-S-acetylthioacetate). To 1 mL of IgG, was added 10 μ L of a 65 mM SATA solution in DMSO, the reaction was mixed under vortex and allowed to react for \sim 30 minutes at room temperature. Excess SATA and reaction by-products were removed on a 5 mL Zeba Desalt spin column (Pierce Chem. Co., Rockford, IL). The sulfhydryl groups were deprotected by reaction with a solution of 0.5 M hydroxylamine HCl in 50 mM sodium phosphate, 25 mM EDTA, pH 7.5, over 2 hours. The sulfhydryl-containing IgG was repurified by a Zeba Desalt spin column. An Ellman's assay¹ was performed to determine the free thiol group concentration present on the IgG₁, which was found to be 2.55×10^{-9} moles, corresponding to 1.6 free thiol groups per antibody molecule. After calculating the number of thiol groups present, a five-fold molar excess (1.28×10^{-8} moles) of a 10 mM DMSO solution of the maleimido-CypHer dye was added for every mole of free thiol. This solution was allowed to react for 2 hours at room temperature and excess unreacted dye was removed by another Zeba Desalt spin column. The conjugate was analyzed by SE-HPLC. The results of this analysis can be seen in **Figure 2**.

Figure 2. The HPLC data shows one peak at both λ_{280} and λ_{598} nanometers, indicating successful conjugation of the CypHer5E dye to IgG₁.



References

1. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys.* 1959 May;82(1): 70-7.
PMID 13650640