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Photostable Cyanine Dye β-Cyclodextrin Conjugates

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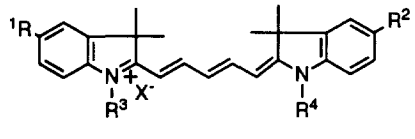
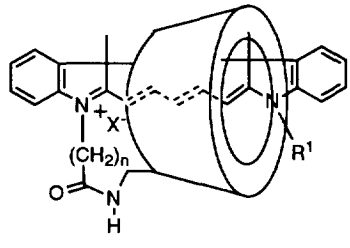
Abstract: Cyanine dye β-cyclodextrin conjugates were synthesized and were demonstrated to possess greatly enhanced photostability in aqueous solution and in live Swiss 3T3 cells. © 1997 Elsevier Science Ltd.

A major and often limiting problem in many fluorescence based diagnostic and imaging techniques is photodegradation of the fluorophore.[1] Upon illumination in aqueous and biological media, reactive oxygen species such as singlet oxygen ($^1O_2$), superoxide and peroxides, and redox active metabolites can degrade the fluorophore.[2] The chemical stability of many compounds is enhanced by their inclusion inside cyclodextrins (CDs) because they are sterically protected from collisions with reactive species.[3] Photobleaching of cyanine dyes in aqueous solution results mainly from the reaction of $^1O_2$ with the polymethine chain of the ground state dye[4] and is suppressed in the presence of CDs.[5,6] We report here our preliminary investigations in using the protective effect of CDs for creating photostable fluorescent labeling reagents.





1a $R^1 = R^2 = H$, $R^3 = Me$, $R^4 = (CH_2)_2CO_2H$
1b $R^1 = R^2 = H$, $R^3 = Me$, $R^4 = (CH_2)_5CO_2H$
1c $R^1 = R^2 = H$, $R^3 = Me$, $R^4 = (CH_2)_{10}CO_2H$
1d $R^1 = R^2 = H$, $R^3 = CH_2CHMe_2$, $R^4 = (CH_2)_2CO_2H$
Cy5 $R^1 = R^2 = SO_3^-$, $R^3 = Et$, $R^4 = (CH_2)_5CO_2H$

2a $R^1 = Me$, n = 2
2b $R^1 = Me$, n = 5
2c $R^1 = Me$, n = 10
2d $R^1 = CH_2CHMe_2$, n = 2

The hydrophobic dyes **1a-d**,[7] the commercial dye Cy5[8] and the **1a**-dextran conjugate (m.w. 40,000, dye/dextran < 1) photobleached in aqueous solution according to pseudo first order kinetics to afford photostabilities for these compounds that were normalized relative to Cy5. (Table 1). The water soluble **1a**-dextran and Cy5 exhibited similar photostabilities suggesting that electronic differences between the sulphonated and non-sulphonated chromophores do not have a significant effect on their relative rates of photobleaching. Dye hydrophobicity increases as the side chain is lengthened causing increased dye aggregation in

aqueous solution. The aggregates photobleach faster than the monomeric dyes[4] and have lower quantum yields of fluorescence (Øf).[4]

Table 1. Relative photostabilities and quantum yields in water.

Compound	Relative Photostability	Øf	Øf x Rel. Photost.
Cy5	1.0	0.20	0.20
1a-dextran	0.95	0.14	0.13
1a	0.53	0.12	0.06
1b	0.41	0.11	0.05
1c	0.13	0.07	0.01
1d	0.53	0.13	0.06
2a	9.1*	0.06	0.55
2b	1.6	0.10	0.16
2c	0.44	0.14	0.06
2d	20*	0.07	1.40

*Pseudo first order bleaching region

β-CD is sterically most compatible for 1:1 complex formation with dyes of the type **1a-d**.[9] Coupling of dyes **1a-d** with 6-amino-6-deoxy-β-CD[10] using DCC/HOBt in DMF afforded the water soluble conjugates **2a-d** after chromatography on Sephadex LH-20 with water methanol 1:1 as eluant. Conjugates **2a** and **2d** showed non-integer order photobleaching (approx. 15 %) followed by a slower pseudo first order process, whereas **2b** and **2c** exhibited pseudo first order photobleaching throughout (Figure 1a). Conjugates **2a-d** are more photostable than their parent dyes due to their reduced tendency to form aggregates and due to the protective effect of

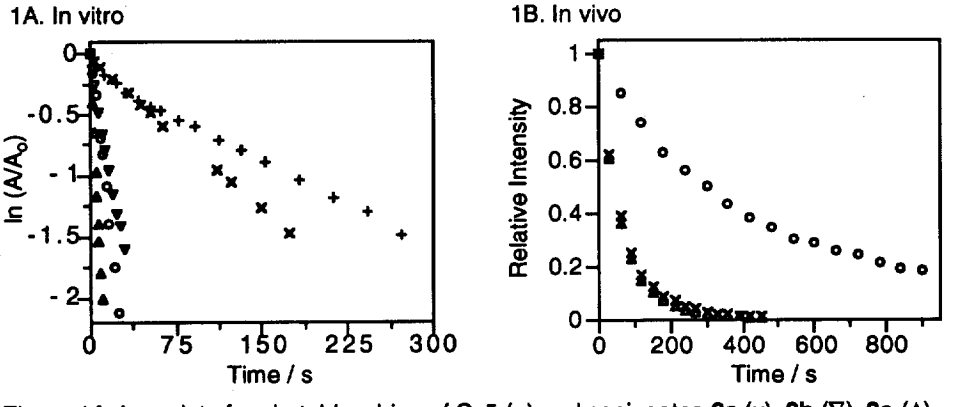


Figure 1A. Log plots for photobleaching of Cy5 (o) and conjugates **2a** (x), **2b** (∇), **2c** (Δ) and **2d** (+) in water; 1B. Fluoresence decay of conjugate **2a** (o), **1a**-dextran (Δ), and Cy5-antibody (x) in Swiss 3T3 cells.

the CD. Photostability is a function of the length of the tether between the CD and the dye, and on the substituent groups of the dye. A longer tether allows the chromophore to adopt more orientations in which it is not protected by the CD. In particular, examination of CPK space filling models indicates that the tethers of conjugates **2b** and **2c** are sufficiently long to allow the chromophore to protrude completely through the secondary face of the cavity and expose it to attack by $^1O_2$. Comparison of the relative photostabilities of **1a**-dextran with **2a** allows separation of the solubility effect on the chromophore stability from that of the CD protective effect. In the case of **2a**, the protective effect of the CD enhances the dye stability 10 fold. For cyanine dye-β-CD complexes, this protective effect has been attributed to a combination of decreased dye sensitized production of the destructive $^1O_2$ and of steric inhibition of the interaction of $^1O_2$ with the chromophore.[5] The low Øf of conjugates **2a** and **2d**, caused by the interaction of the chromophore with the CD, reflects increased non-radiative deactivation of the excited state dye and suggests a shortening of the average excited state lifetime. This would argue in favor of lower $^1O_2$ production being a possibility for the greater photostability of these conjugates. Since conjugates **2a** and **2d** have similar quantum yields, then the difference in photostability between them indicates that factors such as steric protection are also important. Comparison of the product of the relative photostability and Øf indicates that **2a** and **2d** are able to emit approximately 2.7X and 7X, respectively, more photons than Cy5 before they are destroyed.

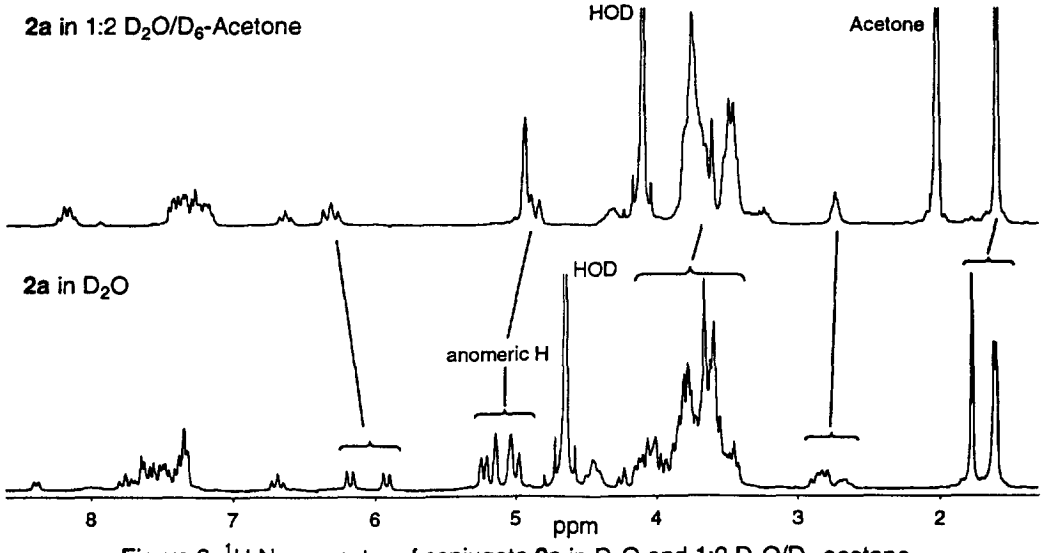


Figure 2. ^{1}H Nmr spectra of conjugate **2a** in D_2O and 1:2 D_2O/D_6-acetone

^{1}H Nmr spectra of conjugates **2a** and **2d** in D_2O are complex. In addition to the lack of symmetry in the CD, the anisotropy of the dye causes splitting of the CD proton signals, which is most easily observed for the anomeric protons. The chromophore protons are also shifted and split relative to those in the non-conjugated dyes. Addition of D_6-acetone to the D_2O solutions

caused the appearance of signals corresponding to those of the free dye and CD moieties. In 1:2 D_2O/D_6-acetone, the spectrum of **2a** more-or-less resembles the sum of the constituent dye and CD spectra indicating that the hydrophobic interaction between the two is broken when the solvent polarity is lowered (Figure 2), whereas for **2d** in this solvent mixture, a ratio of 1:1 was observed for the associated and non-associated structures. The stronger interaction of the chromophore of **2d** with its CD, compared to **2a** is reflected in their relative photostabilities.

Aqueous solutions of conjugate **2a**, **2d**, **1a**-dextran and a non-specific antibody conjugated with Cy5 were microinjected into live Swiss 3T3 cells. The fluorescence intensity of individual cells was recorded at various time intervals during continuous illumination and was used to calculate relative photostabilities. Conjugates **2a** and **2d** were 7X and 8X more photostable, respectively, than **1a**-dextran, which exhibited similar photostability to Cy5-antibody (Figure 1b). Differences in relative photostability between the in vitro and in vivo measurements may result from competition for the CD binding site between the dye and biomolecules in the cells or temperature differences between the in vitro (25 °C) and the in vivo (37 °C) studies. In addition the cellular environment contains many hydrophobic sites that may compete with the CD for binding of the dye. In these sites the dye may be protected less well. Cells injected with conjugates **2a** and **2d** remained viable for several days in culture and underwent normal cell division.

In conclusion, despite the reduced quantum yields of conjugates **2a** and **2d**, we have demonstrated that dye-CD conjugates represent a viable approach to the preparation of photostable fluorescent reagents.

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References

1 Tsien, R.; Waggoner, A.S. *Handbook of Biological Confocal Microscopy*, J.B. Pawley Ed., Plenum Publishing Corporation, New York, **1990**.
2 Foote, C.S. *Science*, **1968**, *162*, 963.
3 Szetli, J.*Cyclodextrins and their Inclusion Complexes*, Academiai Kaido, Budapest, **1982**.
4 Byers, G.W.;. Gross, S.; Henrichs, P.M. *Photochem. Photobiol.*, **1976**, *23*, 37.
5 Matsuzawa, Y.; Tamura, S.-I.; Matsuzawa, N.; Ata, M. *J. Chem. Soc. Faraday Trans.*, **1994**, *90*, 3517.
6 Kusumoto, Y.; Saki, Y.; Kurawaki, J.*Chemistry. Express*, **1989**, *4*, 313.
7 Dyes **1a-d** were prepared using procedures described in Southwick, P.L.; Ernst, L.A.; Tauriello, E.W.; Parker, S.R.; Mujumdar, R.B.; Mujumdar, S.R.; Clever, H.A.; Waggoner, A.S.*Cytometry*, **1990**, *11*, 418.
8 Mujumdar, R.B.; Ernst, L.A.; Mujumdar, S.R.; Lewis, C.J.; Waggoner, A.S. *Bioconjugate Chemistry*, **1993**, *4*, 105.
9 Kasatani, K.; Ohashi, M.; Sato, H. *Carbohydr. Res.* **1989**, *192*, 197.
10 Melton, L.D.; Slessor, K.N. *Carbohydr. Res.*, **1971**, *18*, 29.

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