Asymmetric trimethine 3*H*-indocyanine dyes: efficient synthesis and protein labeling†

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We present an efficient method to synthesize three new asymmetric trimethine cyanine dyes containing only one carboxylic acid group for bioconjugation. Two of them have better protein labeling performance than other conventional cyanine dyes due to their particular structure design.

Intensive research efforts have been devoted to the design and synthesis of novel water-soluble fluorescent organic dyes for life science applications especially protein research during the past decade.[1,2] Among them, 3*H*-indocyanine dyes with multi-sulfo groups have received considerable attention and were widely used as fluorescent labeling compounds for proteins. This is because they have large molar extinction coefficients, moderate-to-high fluorescence quantum yields, and a broad wavelength tunability.[3] Usually these cyanine dyes have two or four sulfo groups symmetrically on the two ends of their molecular structure. And two active functional groups (like carboxylic acid) are often introduced for bioconjugation, as seen in a commercially available dye **1a** (Scheme 1). Its more stable alterative, **1b**, was reported by our group previously.[4,5]

As well known, single labeling site is more desirable for protein labeling to obtain specific-position target and to avoid protein crosslinking.[1,6,7] However, in many cases it was found difficult to synthesize and purify asymmetric cyanine dyes with multi-sulfo groups and single labeling site, such as one carboxylic acid group. For example, one asymmetric cyanine dye **1c** (Scheme 2) with this kind of structure was prepared in a very low yield (5%).[8]

Here we developed a stepwise synthesis route for three new asymmetric trimethine 3*H*-indocyanine (Cy3) dyes **1d–f** with multi-sulfo groups and one carboxylic acid group with much better yields. As so far, no detailed investigation has been reported about protein labeling efficiency of such asymmetric cyanine dyes. This work highlights excellent protein labeling performance of two asymmetric dyes **1e–f** because of their particular structure.

These asymmetric dyes could be obtained by mixing two different quaternary salts **2a–e** and a coupling reagent *N*, *N*′-diphenylformamidine. But the purification is difficult and the yield is horrible. In this study, dyes **1c–d** were synthesized by a stepwise route (Scheme 2). *N*, *N*′-diphenylformamidine (1.2 eq.) reacted with one quaternary salt **2a** (1.0 eq.) to afford a hemicyanine intermediate **3**. Then another quaternary salt **2** was added to react with **3** in acetic anhydride to produce an asymmetric dye . The adding sequence of different quaternary salts **2** has a great influence on the total yield of dyes. We found that in the first step, adding quaternary salt **2a–c** instead of **2d–e** can improve apparently the yield of the desired asymmetric dyes, which is because the electron-withdrawing group on the 3*H*-indolium ring of **2a–c** can reduce the reactivity of quaternary salt **2** with the formed hemicyanine intermediate **3** to form undesired symmetric dyes.

As shown in Scheme 2, new asymmetric dyes **1e** (35%) and **1f** (42%) were obtained by using the quaternary salt **2a** (with low reactivity) as the first quaternary salt and **2d** or **2e** (with relatively high reactivity) as the second quaternary salt. Their yields are greatly improved as compared with reported asymmetric Cy3 dyes **1c** (5%) in the literature,[8] and better than the yields of **1d** (21%) which was obtained from the reaction with quaternary salt **2b** or **2c** (with low reactivity) as the second quaternary salt.

After purified on C18-RP column using methanol–water mixture as eluent, these asymmetric dyes were converted to their NHS active ester for protein labeling (ESI Scheme S1†). The NHS esters were used without further purification due to easy to be contaminated and deactivated *via* hydrolysis.

Bovine serum albumin (BSA) was chosen as the protein for bioconjugation. After labeling reaction, the obtained dye-BSA

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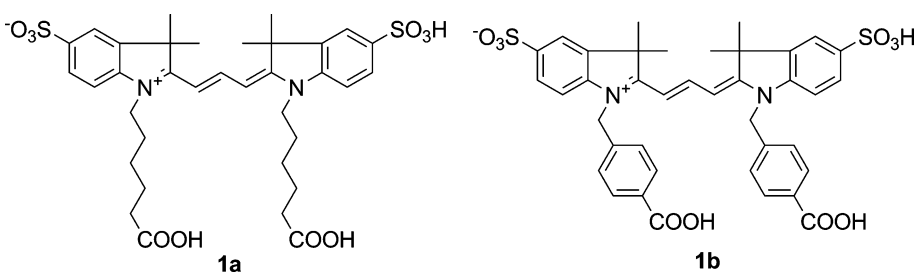


Scheme 1 Two symmetric trimethine cyanine dyes.

Table 1 Spectral properties of dye-BSA conjugates in water

Entry	$\lambda_{abs}/\lambda_{em}$/nm	Stokes shifts/nm	Φ^a $_{dye\text{-}BSA}$	$\Phi_{dye\text{-}BSA}/\Phi_{dye}$
1a-BSA	555/570	15	0.32	1.6
1b-BSA	556/572	16	0.35	2.5
1c-BSA	553/569	16	0.34	1.9
1d-BSA	551/570	19	0.22	2.2
1e-BSA	554/572	18	0.39	3.5
1f-BSA	555/571	16	0.36	2.8

[a] The fluorescence quantum yields were determined in reference to Rhodamine B in ethanol ($\Phi = 0.97$)[9]. λem: 554 nm, error *ca.* ±10%.

conjugates were separated by HPLC to remove the excess dyes, and their spectral properties were tested (see Table 1).

All dye-BSA conjugates were found to have a slight red-shift (4–7 nm) in their absorption and emission spectra when compared with the free dyes (Table 1 and ESI Table S1†).[10] Moreover the fluorescence was significantly enhanced. The fluorescence quantum yields of dye-BSA conjugates reach about 0.39 which are 1.6–3.5 folds of the free dyes' in water. Fig. 1 shows the emission spectra of **1f**-BSA and **1b**-BSA conjugates and their free form. The fluorescence-enhancing effect might be resulted from the rigid microenvironment when the dyes are conjugated to proteins.[8] The fluorescence quantum yields of dyes-BSA conjugates are similar and around 0.35, expect that of **1d**-BSA.

Dye/protein (D/P) ratios are usually used to evaluate labeling efficiency of dyes. After these Cy3 dyes were labeled on BSA under the same conditions, their D/P ratios were calculated based on the absorbances of free dyes and dye-BSA bioconjugates. The results show that the D/P of dye **1e** (2.5) and **1f** (2.2) were about 2–3 folds of that of dye **1a–d** (Table 2).

To further confirm the better protein labeling performance of dye **1e–f**, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out for all these dye-BSA conjugates. The LOD (limit of detection) value is used for quantification of the minimum amount of protein for the specified method.[11] As expected, new dyes **1e–f** do possess the best LOD, **1a–b** the second, but **1c–d** are poor (Table 2). Fig. 2 shows the comparison of **1f**-BSA and **1b**-BSA on gel photographs.

Table 2 D/Ps and LODs of dyes for BSA by SDS-PAGE

Entry	**1a**	**1b**	**1c**	**1d**	**1e**	**1f**
D/P	1.5	1.4	0.9	0.8	2.5	2.2
LOD/ng[a]	100	100	500	500	50	50

[a] BSA denaturation: pH = 8.7, SDS 2.5%, boiling for 10min; Optimal Cy3 labeling conditions were obtained based on experiments of dye **1b** (see ESI Figure S8 and Figure S9†): pH = 8.7, at 30 °C for 30 min.

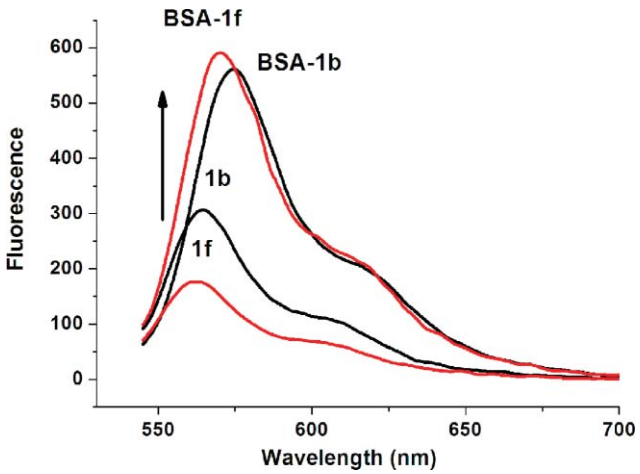
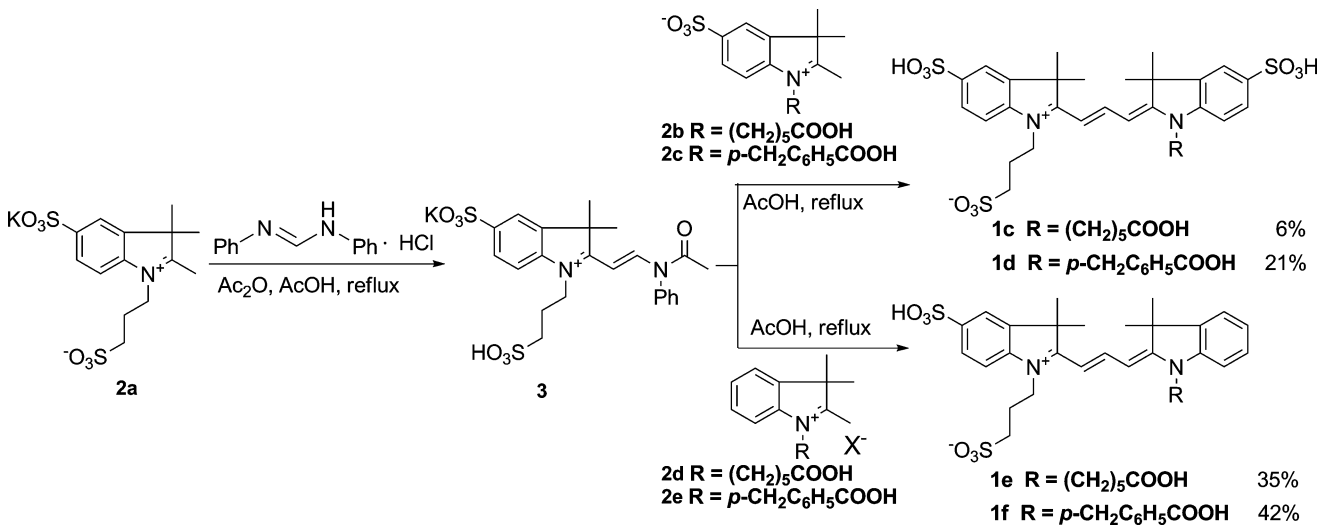


Fig. 1 Fluorescence enhancement after labeling BSA with dye **1f** (red) and **1b** (black) in water (when the absorption maxima ($A_{\lambda max}$) of these samples were about 0.095).

Because no significant difference can be found in the fluorescence quantum yield of all these dye-BSA conjugates (see Table 1), we speculate that the molecular structure effect claims the better protein labeling performance of dyes **1e–f** than that of **1c** and **1d**. Both **1e**-NHS and **1f**-NHS have a hydrophobic end at the NHS ester reaction center; whereas **1c**-NHS and **1d**-NHS have a hydrophilic sulfo-group at the end with the NHS ester (Fig. 3). The hydrophilic sulfo-group might have a repulsing effect from the protein chain (with carboxylic groups), which retards the labeling reaction between dyes and protein.



Scheme 2 Synthesis route of the asymmetric trimethine cyanine dyes.

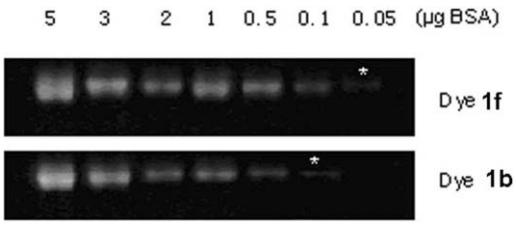


Fig. 2 Gel photographs showed the LODs of dye **1f** and **1a**.

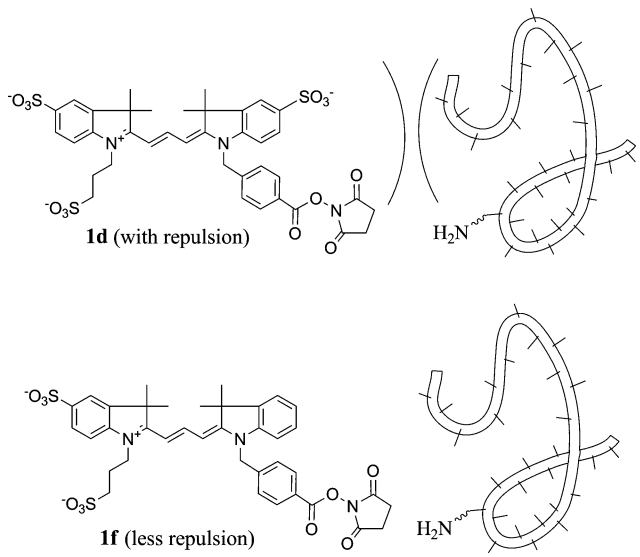


Fig. 3 Repulsion effect between the dye and the protein chain.

Both dye **1e** and **1f** exhibit excellent protein labeling performance. But dye **1f** should have better photostability since it contains one *N*-*p*-carboxybenzyl (*N*-*p*-$CH_2C_6H_4COOH$) on the indole ring.[4] So we choose to quantify the LOD of dye **1f** in a more accurate way by HPLC with a florescence detector excited at $\lambda_{abs-max}$ 554 nm. As shown in Fig. 4, **1f**-NHS could label BSA with a concentration as low as 20 nM by HPLC. In addition,

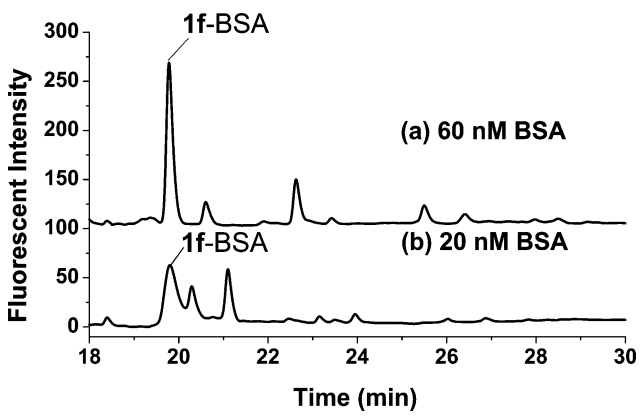


Fig. 4 Analysis of **1f**-NHS labeled BSA with low concentration (a) 60 nM BSA and (b) 20 nM BSA by HPLC with fluorescence detector (excited at 554 nm and detected at 572 nm). Experimental conditions were mentioned in ESI.†

with the decrease in BSA concentration from 60 nM to 20 nM, the fluorescent signal of **1f**-BSA decreased about 3 times. In other words, the fluorescent signal was proportional to BSA concentration in this range. With fluorescence detector and under the optimized labeling conditions, the LOD of **1f** could decrease to 4.8×10^{-10} M (or 0.6 ng), considering that the signal-to-noise ratio of should be above 3 to check the LOD of HPLC.[12] For comparison, by the same method the LOD of **1b** was detected and found to be 8.7×10^{-10} M (or 1.1 ng) (see ESI Figure S9†), about 2-fold higher than that of **1f**.

In conclusion, a series of asymmetric Cy3 dyes were synthesized. The stepwise route affords an asymmetric dye with yield 42%. SDS-PAGE and HPLC with fluorescence detector are applied to evaluate the labeling performance of the Cy3 dyes on BSA. Under the optimal conditions of SDS-PAGE, the LOD of **1f**-BSA is as low as 50 ng, which can be directly observed by naked eyes under UV light. And in HPLC experiments, the LOD could decrease to 4.8×10^{-10} M (or 0.6 ng). The excellent labeling performance of **1f** is attributed to its particular structure with the hydrophobic groups on one end and hydrophilic sulfo-groups on the other end. We believe dyes **1e–f** will be found beneficial in the bioassay applications.

Acknowledgements

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