Journal of Photochemistry and Photobiology A: Chemistry 210 (2010) 168–172

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology A: Chemistry

journal homepage: www.elsevier.com/locate/jphotochem

Novel asymmetric Cy5 dyes: Synthesis, photostabilities and high sensitivity in protein fluorescence labeling

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ARTICLE INFO

Article history:
Received 30 October 2009
Received in revised form
13 December 2009
Accepted 4 January 2010
Available online 6 January 2010

Keywords:
Cy5
Cyanine dye
Photophysical property
Photostability
Fluorescent labeling
High performance liquid chromatography

ABSTRACT

Several novel water-soluble asymmetric pentamethine cyanine dyes were synthesized. The maximum absorption and emission wavelengths of the dyes in different solvents were in the range from 647 to 665 nm and exhibited negative solvatochromism with increasing solvent polarity. The fluorescence quantum yields of the dyes were about 0.1 in water, and were obviously higher than those of hydrophobic cyanine dyes. Dyes with *N*-benzyl groups and *N*-sulfo-groups displayed greater photostability than dyes with *N*-carboxypentyl groups in water. The limit of detection of dye **5a** for BSA was 1.2×10^{-8} mol L^{-1} by high performance liquid chromatography with fluorescent director about 100-fold lower than that by UV detection (1.0×10^{-6} mol L^{-1}). Therefore, Dye **5a** could be used to improve photostability and detection sensitivity in protein analysis.

1. Introduction

In the post-genome sequencing era, there has been more and more attention paid to protein analysis. Until now, fluorescence detection has proved to be one of the most sensitive methods for protein analysis [1,2]. Fluorescence detection combined with electrophoresis [3,4] and high performance liquid chromatography (HPLC) techniques enable qualitative and quantitative analysis of proteins. However, most proteins with important biological functions, such as the drug targets and biomarkers, are of an extremely low concentration [5]. Therefore, the study of more suitable fluorophores for the improvement on detection sensitivity for proteins is an imperative task.

Cyanine dyes play an indispensable role in biomedical applications [6,7], particularly in fluorescence detection of antibodies and DNA [8], the imaging of biological targets in vivo [9], and fluorescent labeling compounds for proteins [10,11]. This is due to their excellent spectral properties, including large molar extinction coefficients and broad wavelength tunabilities.

In order to effectively reduce the background signal arising from auto-fluorescence of the biological matrix and light scattering, longwavelength cyanine dyes (>600 nm or within the near infra red region) have been developed. Increasing the length of the conjugated chain of the cyanine dyes is the main approach to impart the desired red shift; however, this reduces the photostability [12].

In our previous work, we firstly employed rigid *N*-*p*-carboxybenzyl (*N*-*p*-$CH_2C_6H_4COOH$) as substituents in 3*H*-indolenine trimethine cyanine dyes (Cy3) [13], and 3*H*-indolenine heptamethine cyanine dyes (Cy7) [14], that not only improved the photostability, but also increased the yields of intermediates and products. The properties of pentamethine cyanine dyes (Cy5) of 3*H*-indolenines, however, remain unreported. The bio-labeling of the dyes to proteins via covalent conjugations include the activation of *N*-*p*-carboxybenzyl in the dyes and the reaction of the activated dyes to reactive groups (such as $-NH_2$) on proteins in aqueous buffer solutions under mild conditions. Sulfo-groups are important for this kind of application because they prevent protein precipitation during the derivatization procedures [15,16].

In this paper, we describe the synthesis of the water-soluble Cy5 dyes, the relationship between the molecular structure and photostability and labeling performance on BAS. The structures of water-soluble Cy5 dyes were shown in Fig. 1. Compared with well-

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doi:10.1016/j.jphotochem.2010.01.002

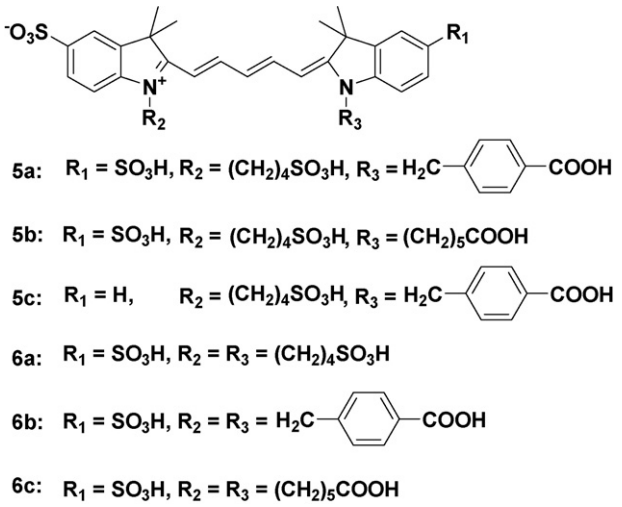

Fig. 1. Structures of water-soluble Cy5 dyes.

known symmetric cyanine dye **6c**, the asymmetric dyes (**5a** and **5b**) contain only one active group on their molecules, and should benefit from quantitative protein labeling and purifying. All results demonstrated that asymmetric dye **5a** possesses better photostability, which can be a good fluorescent labeling reagent for protein labeling.

2. Experiments

2.1. Instruments and materials

Mass spectral determinations were taken on HP1100 API-ES mass spectrometer. NMR spectra were recorded on a Varian 400 MHz NMR spectrometer (USA). Chemical shifts are expressed in parts per million from D_2O (δ_H = 4.79) [17]. Fluorescence measurements were performed on a PTI-C-700 Felix and Time-Master system. Visible spectra were measured on a HP-8453 spectrophotometer. HPLC experiments were performed on the Waters 2695-2996-2475. Purification of dyes was performed by conventional column chromatography with C18-RP absorbent (Sinochrom C18, 40–75 mesh, 10 nm, 280 $m^2\ g^{-1}$, Dalian Elite Company, China). Deionized water was redistilled before use, and acetonitrile was of chromatographic grade. Other chemicals used for the experiments were of analytical grade.

2.2. Synthesis

The synthetic routes of Cy5 dyes were shown in Fig. 2. 2,3,3-Trimethyl-3*H*-indolenine and 2,3,3-trimethylindolenine-5-sulfonate (**2**) were obtained as starting materials by conventional Fisher 3*H*-indole synthesis [18].

Intermediates of 3*H*-indolium quaternary salt **3** were synthesized from the quaternization of **2** with 1,4-butane sultone, 6-bromohexanoic acid or *p*-(chloromethyl) benzoic acid, respectively, and were used in further experiments without additional purification. The yields of intermediates **3a**, **3b**, **3c** and **3d** were 78%, 58%, 39% and 80%, respectively. Symmetric Cy5 dyes, as reference dyes, were synthesized according to the previous procedure [16].

Dye **6a**: Yield 64%. ^{1}H NMR (400 MHz, D_2O): δ 1.48–1.69 (m, 8H, $4CH_2$), 1.78 (s, 12H, $C(CH_3)_2$), 2.79 (m, 4H, $CH_2SO_3^-$), 3.94 (m, 4H, N–CH_2), 6.12 (m, 2H, CH=CH), 6.39 (d, 1H, J = 13.6 Hz, CH=CH), 7.19 (m, 2H, Ar–H), 7.59 (m, 2H, Ar–H), 7.64 (s, 2H, Ar–H), 7.84 (t, 2H, J = 13.2 Hz, CH=CH). API-ES-MS, m/z: 261.2 $[M-3H]^{3-}$.

Dye **6b**: Yield 69%. ^{1}H NMR (400 MHz, D_2O): δ 1.54 (s, 12H, $C(CH_3)_2$), 5.19 (s, 4H, N–CH_2–Ar), 6.02 (d, 2H, J = 13.6 Hz, CH=CH), 6.19 (m, 1H, CH=CH), 7.04–7.76 (m, 14H, Ar–H), 7.82 (t, 2H, J = 13.6 Hz, CH=CH). API-ES-MS, m/z: 390.6 $[M-2H]^{2-}$.

Dye **6c**: Yield 60%. ^{1}H NMR (400 MHz, D_2O): δ 1.28–1.66 (m, 12H, $6CH_2$), 1.74 (s, 12H, $C(CH_3)_2$), 2.79 (m, 4H, CH_2COOH), 3.93 (m, 4H, N–CH_2), 6.02 (m, 2H, CH=CH), 6.44 (d, 1H, J = 13.2 Hz, CH=CH), 7.49 (m, 2H, Ar–H), 7.64 (m, 2H, Ar–H), 7.76 (s, 2H, Ar–H), 7.94 (t, 2H, J = 13.2 Hz, CH=CH). API-ES-MS, m/z: 740.6 $[M-2H]^{2-}$.

2.2.1. Hemicyanine intermediate *4*

The quaternary salt **3a** (375 mg, 1 mmol) and malonaldehyde dianil hydrochloride (310 mg, 1.2 mmol) were dissolved in a mixture of acetic acid (5 mL) and acetic anhydride (5 mL), and then heated to reflux. The reaction was monitored by thin-layer chromatography (TLC). Extended heating produced some symmetrical dye (<5%). After 50 min, the mixture was cooled to room temperature and diluted with diethyl ether. The supernatant fluid was removed by decantation. The brown powder thus obtained was simply separated by flash-column.

2.2.2. Asymmetrical dyes

A solution of crude hemicyanine intermediate **4** (585 mg, 1 mmol) and quaternary salt **3b**, **3c** or **3d** (1 mmol) in acetic anhydride (5 mL) was heated to 120 °C for 40 min. The reaction was monitored by TLC. The mixture was cooled to room temperature and diluted with diethyl ether. After filtration, the crude dye was chromatographied in a C18-RP column using a methanol–water mixture as the eluent.

Dye **5a**: Yield 56%. ^{1}H NMR (400 MHz, D_2O): δ 1.53 (m, 4H, $2CH_2$), 1.74 (s, 12H, $C(CH_3)_2$), 2.74 (m, 2H, $CH_2SO_3^-$), 3.92 (s, 2H, N–CH_2), 5.18 (s, 2H, N–CH_2–Ar), 6.02 (m, 2H, CH=CH), 6.26 (t, 1H, J = 13.2 Hz, CH=CH), 7.02–8.42 (m, 12H, Ar–H), 7.76 (m, 2H, CH=CH). API-ES-MS, m/z: 391.1 $[M-2H]^{2-}$.

Dye **5b**: Yield 30%. ^{1}H NMR (400 MHz, D_2O): δ 1.27–1.67 (m, 10H, $5CH_2$), 1.68 (s, 12H, $C(CH_3)_2$), 2.08 (m, 2H, CH_2COOH), 2.81 (m, 2H, $CH_2SO_3^-$), 3.94 (m, 4H, N–CH_2), 6.14 (m, 2H, CH=CH), 6.46 (t, 1H, J = 12.8 Hz, CH=CH), 7.19 (m, 2H, Ar–H), 7.62 (m, 2H, Ar–H), 7.69 (d, 2H, J = 5.2 Hz, Ar–H), 7.84 (m, 2H, CH=CH). API-ES-MS, m/z: 381.0 $[M-2H]^{2-}$.

Dye **5c**: Yield 64%. ^{1}H NMR (400 MHz, D_2O): δ 1.71 (m, 4H, $2CH_2$), 1.73 (s, 12H, $C(CH_3)_2$), 3.17 (m, 2H, $CH_2SO_3^-$), 4.15 (m, 2H, N–CH_2), 5.36 (s, 2H, N–CH_2–Ar), 6.37 (m, 2H, CH=CH), 6.42 (m, 1H, CH=CH), 7.13–7.78 (m, 11H, Ar–H), 8.34 (t, 2H, J = 12.8 Hz, CH=CH). API-ES-MS, m/z: 703.2 $[M-H]^-$.

2.2.3. Synthesis of the succinimidyl esters *7* of Cy5 dyes

The dye with carboxyl group was dissolved in dry *N*,*N*-dimethylformamide (DMF, 2 mL/100 mg of the dye). *N*,*N*′-dicyclohexyl carbodiimide (DCC, 5 eq./carboxyl group) and *N*-hydroxysuccinimide (NHS, 10 eq./carboxyl group) was added. The mixture was left at room temperature for 10 h. After diluting the mixture with dry ethyl acetate, the supernatant was centrifuged and collected. By TLC, a nearly 100% yield of the active succinimidyl esters of Cy5 dyes (Cy5-NHS) was obtained. Because active esters easily become deactivated via hydrolysis, the products were prepared on the spot and used immediately without further purification.

2.3. Determination of quantum yield

The corresponding fluorescence quantum yields were calculated relative to a standard solution of Rhodamine B in ethanol

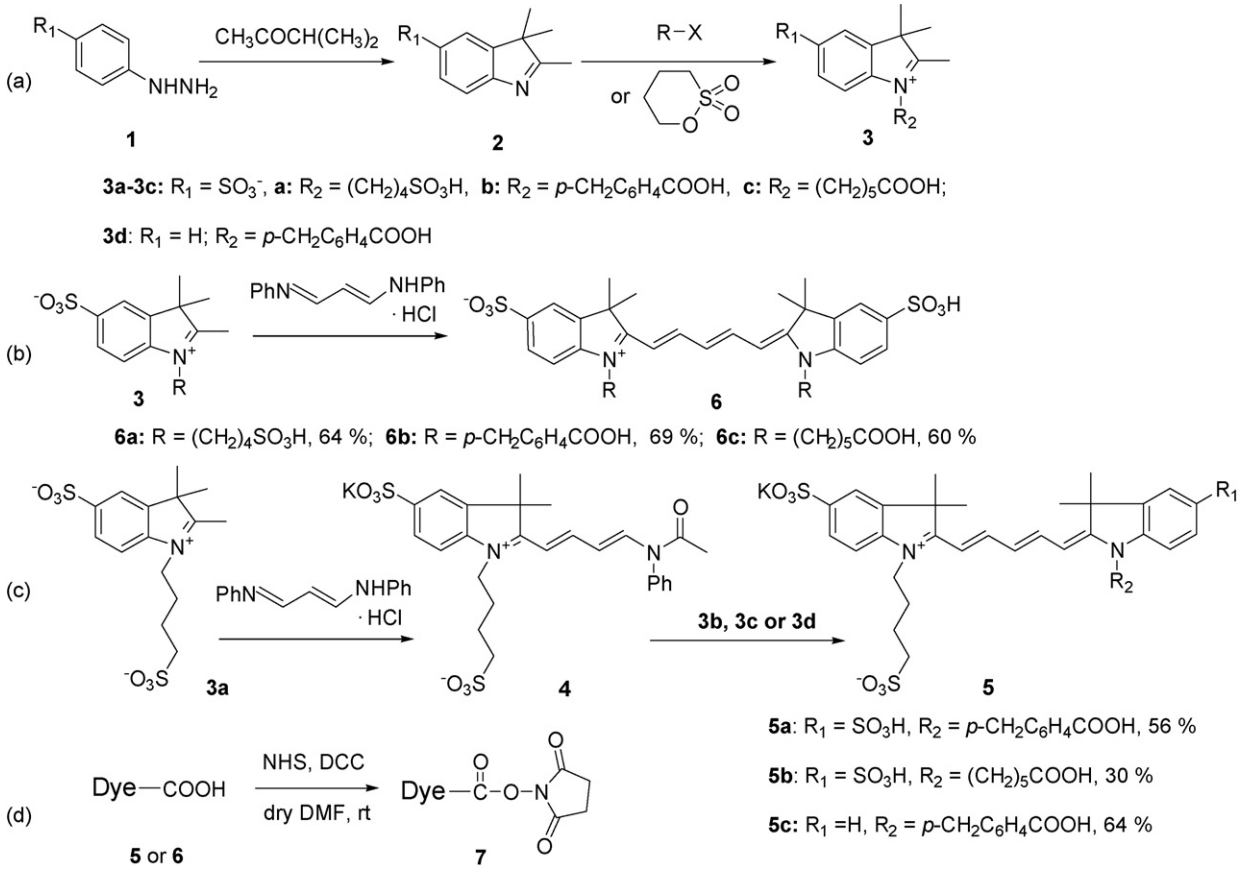

Fig. 2. Synthetic routes of Cy5 dyes: (a) intermediates, (b) symmetric dyes, (c) asymmetric dyes, and (d) succinimidyl esters of Cy5 dyes.

(Φ = 0.56) [19] and were determined using the formula (1) [20]:

$$\Phi_x = \Phi_s \left(\frac{F_x}{F_s}\right)\left(\frac{A_x}{A_s}\right)\left(\frac{\lambda_{exs}}{\lambda_{exx}}\right)\left(\frac{n_x}{n_s}\right)^2 \quad (1)$$

where Φ is quantum yield, F is integrated area under the corrected emission spectrum, A is absorbance at the excitation wavelength, λ_{ex} is the excitation wavelength, n is the refractive index of the solution used in measurement [21] and the subscripts x and s denote test and the standard, respectively.

2.4. Photostability

The photostability tests were carried out in quartz cells (10 mm in width) where sample solutions were irradiated with a 500 W Iodine–tungsten (I/W) lamp at room temperature. The distance between the cells and the lamp was 250 mm. The solutions of the dyes (1×10^{-5} mol L^{-1}) in water were radiated and the irreversible bleaching of the dyes at the absorption peak was monitored as a function of time.

Table 1
Spectra properties of the Cy5 dyes in the different solvents.

Dye	Solvent	$\lambda_{ab}/\lambda_{em}$ (nm)	Φ	ε($\times 10^5$ mol^{-1} cm^{-1} L)	Stocks shifts (nm)
	DMF	660/678	0.15	0.95	18
5a	Methanol	650/670	0.13	1.14	20
	Water	647/664	0.10	1.12	17
	DMF	660/679	0.16	0.97	19
5b	Methanol	650/668	0.13	1.07	18
	Water	648/664	0.10	1.14	16
	DMF	656/674	0.14	0.95	18
5c	Methanol	649/667	0.11	1.12	18
	Water	645/662	0.09	1.28	17
	DMF	660/678	0.14	1.03	18
6a	Methanol	649/670	0.13	1.16	21
	Water	647/663	0.09	1.15	16
	DMF	662/680	0.16	1.02	18
6b	Methanol	653/672	0.12	1.13	19
	Water	648/663	0.11	1.58	15
	DMF	660/677	0.14	1.12	17
6c	Methanol	651/668	0.11	1.15	17
	Water	648/665	0.10	1.23	17

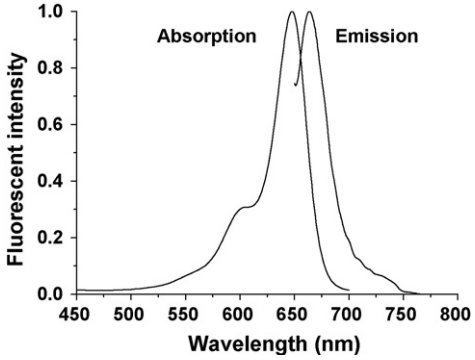

Fig. 3. Absorption and fluorescence emission spectra of dye **5a** in water.

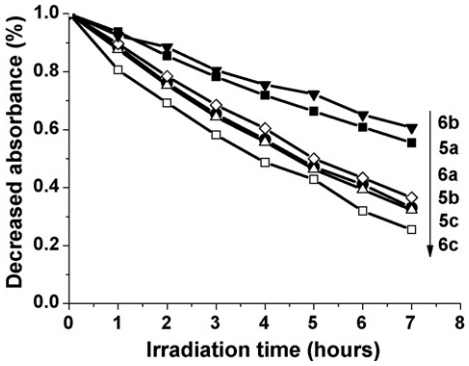

Fig. 4. Photofading behavior of the Cy5 dyes in water.

2.5. General protein labeling procedures

An aqueous solution of BSA (14 μmol L^{-1}, 60 μL) was mixed with 2.5% (w/v) SDS aqueous solution (60 μL) and borate buffer (50 mmol L^{-1}, 30 μL, pH 9.0). After the mixture was stirred at 50 °C for 30 min, Cy5-NHS in dry DMF (8.5 mmol L^{-1}, 10 μL) was added, and then stirred at 50 °C for 30 min in the dark. The sample was further diluted with borate buffer (pH 9.0) and then cooled to 25 °C. Subsequently, 10 μL aliquot of the derived protein mixture was injected, and further analyzed by HPLC.

2.6. HPLC

Column 250 × 4.6 mm (C8, 5 μm and 30 nm); eluent A, 100% water with 0.1% TFA; eluent B, 95% acetonitrile with 0.1% TFA; gradient conditions: 0–27 min, 25–80% B, 27–35 min: 80–80% B; flow rate: 1.0 mL min^{-1}; the column temperature: ambient temperature. Detector: fluorescence detection: λ_{ex} 648 nm, λ_{em} 664 nm; UV detection: λ_{abs} 214 nm.

3. Results and discussion

3.1. Spectral properties

The absorbance and fluorescent properties of water-soluble Cy5 dyes in different solvents are summarized in Table 1. Typical absorption and emission spectra of Cy5 dye were shown in Fig. 3. The maximum absorption and emission wavelengths of dyes in different solvents were in the range from 647 to 680 nm. The dyes exhibited a strong absorption in red visible region with high molar extinction coefficients (ε) in solutions (10^5 mol^{-1} cm^{-1} L).

As shown in Table 1, all these dyes exhibited negative solvatochromism, with a blue shift of the absorption and emission maximum with increasing solvent polarity (e.g. **5a**: λ_{abs} in DMF is 660 nm and 647 nm in water; λ_{em} in DMF is 678 nm and 664 nm in water). The effect of the solvent polarity on λ_{max} could be illustrated by interactions between the dye molecules and the solvents, as the interactions make the ground state of dye more stable by forming hydrogen bonds [22].

Although the fluorescence quantum yields (Φ) of these water-soluble dyes were lower in polar solvents, those were also moderate (0.09–0.11) in water, obviously higher than those of hydrophobic dyes (*ca.* 0.01 in water). The introduction of multi-sulfo-groups could significantly enhance water-solubility, reduce aggregation and fluorescence self-quenching in aqueous media.

3.2. Photostability

Good photostability is an important requirement for the application of fluorescent dye in bio-labeling and bio-imaging. In order to find the relationship between structure and photostability of cyanine dyes, the photostabilities of the dyes with different substituents on *N*-position of 3*H*-indolenine were studied in water. From Fig. 4, it could be shown that after 7 h of irradiation, dye **6c** showed 74% photofading, and dyes **5b**, **5c** and **6a** faded 64–68%. However, dyes **5a** and **6b** showed about only 39–44% decrease in maximal absorbance. The photostabilities of the dyes can be placed in the order: **6b** > **5a** > **6a** > **5b** > **5c** > **6c**. The photostability of asymmetric dye **5c** was relatively lower because it contained only one sulfo-group on its 3*H*-indolium ring, and sulfo-groups are strong electron-withdrawing groups and can reduce the electron density of the dye's pentamethine chain.

The rate constants of photofading (k) for the Cy5 dyes could be calculated according to the formula (2) [14]:

$$\ln\left(\frac{A_0}{A_t}\right) = kt \quad (2)$$

where A_0 is the absorbance in maximal wavelength before the irradiation and A_t is the absorbance in maximal wavelength after the irradiation. The rate constants of the photoreaction based on the experimental data are shown in Table 2. The data showed that except for dye **5c**, the dyes with benzyl ring in the *N*-substituents (**5a** and **6b**) were more stable than those with *N*-alkyl substitution (**5b**, **6a** and **6c**). In addition, for the dyes with *N*-alkyl substituents, *N*-sulfonatobutyl groups were better than *N*-carboxypentyl groups for stability. The photostability of commercial dye **6c** with two *N*-carboxypentyl groups was worst among these dyes. For example, the photofading constant k of *N*-carboxybenzyl dye (**5a**) was less than 60% of the k of *N*-sulfonatobutyl dye (**6a**) and less than half of the k of *N*-carboxypentanyl dye (**6c**).

Therefore, the effect of different *N*-substituents on the photostability of the dyes can be placed in the order: carboxylbenzyl group > sulfonatobutyl group > carboxylpentanyl group. The main reason for this may be steric hindrance of benzyl and sulfo-groups preventing the attack of singlet oxygen, thus improving the photostability of Cy5 dyes.

3.3. Fluorescent labeling on BSA

Considering its high yield, good photostability and water-solubility, novel dye **5a** was converted to its NHS active ester, and then used to label the standard protein BSA (Fig. 5).

Table 2
Rate constants of photofading.

Molecule	**5a**	**5b**	**5c**	**6a**	**6b**	**6c**
Rate constants k ($\times 10^{-3}$ mol min^{-1})	1.41	2.61	2.68	2.41	1.17	3.14

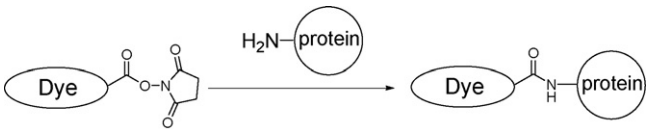

Fig. 5. Protein derivatization with Cy5-NHS.

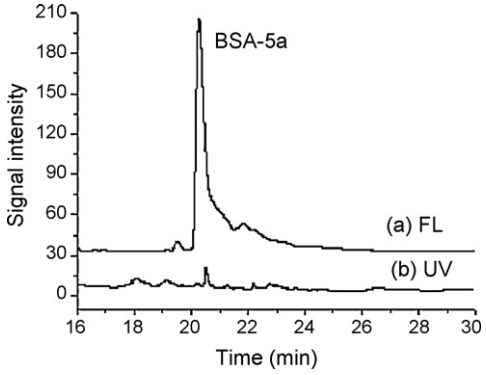

Fig. 6. Analysis of **5a**-NHS labeled BSA by HPLC (a) with fluorescent detector (λ_{ex} 648 nm, λ_{em} 664 nm), (b) with UV detector (λ_{abs} 214 nm). Experimental conditions were mentioned in Experimental Procedures.

It should be noted that the limit of detection (LOD) here refers to the minimum amount of protein that could be derivatized with a detection signal-to-noise ratio of 3 [23]. In this paper, the LODs were evaluated by HPLC with fluorescent detector and UV detector. In our previous work [24], the LOD of commercial fluorescein isothiocyanate (FITC) for BSA was 0.4×10^{-6} mol L^{-1}. In comparison with isothiocyanate-containing dyes (e.g. FITC), NHS-carboxyl-containing Cy3 dyes had relatively high reaction activity and selectivity in protein labeling under mild conditions.

As shown in Fig. 6, the fluorescent signal of **5a**-BSA could be clearly detected when the UV signal of BSA was quite weak. Therefore, compared to results using UV detection, more proteins with low concentrations could be labeled by **5a**-NHS, resulting in improved detection sensitivity for protein analysis. For BSA, the LOD of dye **5a** as measured by fluorescent detection was 1.2×10^{-8} mol L^{-1}, about 100 times lower than that by UV detection (1.0×10^{-6} mol L^{-1}). These results further suggested that a high sensitivity detection of proteins could be easily achieved after **5a**-NHS derivatization and detection by HPLC with a fluorescence detector, especially for those at a low concentration.

4. Conclusion

In this paper, novel water-soluble asymmetric Cy5 dyes were synthesized and their photostability in water were investigated to ensure the good reproducibility of protein fluorescent labeling. It is demonstrated that the benzyl group and sulfo-group on the *N*-position of 3*H*-indolium pentamethine cyanine dyes improved the photostability in aqueous solutions. Furthermore, the LOD of dye **5a**-NHS for BSA was 1.2×10^{-8} mol L^{-1} as measured by HPLC using a fluorescent detector. Compared with UV detection, the sensitivity of detection was increased about 100 times by fluorescent labeling of dye **5a**. All results demonstrated that dye **5a** was a good fluorescent labeling reagent to achieve high sensitive detection for low concentration proteins.

Acknowledgements

This work was supported by NSF of China (20725621 and 20706008), National Basic Research Program of China (2009CB724706), Ministry of Education of China (Program for Changjiang Scholars and Innovative Research Team in University, IRT0711, and Cultivation Fund of the Key Scientific and Technical Innovation Project, 707016).

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