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ORIGINAL PAPER

Novel Heptamethine Cyanine Dyes with Large Stokes' Shift for Biological Applications in the Near Infrared

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Abstract A series of novel functionalized, water-soluble, pH-unsensitive, highly photostable heptamethine cyanine dyes (HCDs) has been synthesized. The aim of the synthesis was to obtain novel effective probes for fluorescence detection in the near infrared. Synthesis and characterization of a special HCD with large Stokes' shift (>100 nm), bioconjugation to IgG and effect of pH upon the new structure are presented.

Keywords Cyanine · NIR dyes · Bioconjugation · Large Stokes' shift

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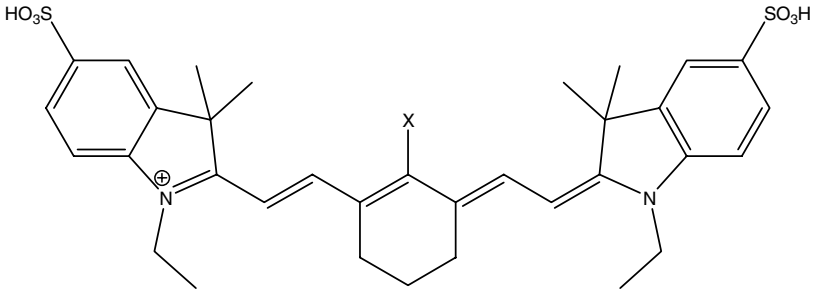
Introduction

The use of fluorescent probes for biological applications is, nowadays, an established methodology. Sulfoindocyanine dyes, in particular, are widely applied, since they are water-soluble and their carboxylic acid derivatives easily modifiable to succinimidyl esters. Although cyanine dyes offer spectral features ranging from visible to NIR, the latter region is still poorly exploited. New promising techniques as fluorescence *in-vivo* imaging are actually seeking for NIR dyes, to overcome the problem of biomolecules and tissues self fluorescence. Interesting chances could be given by the work of Mullen about perylene-based DNA nanoscale objects, which offer very high quantum yield and not negligible emission wavelength (around 650 nm). This work is DNA related and devoted to molecular electronics, and has not yet been investigated for diagnostics/life sciences purposes [1, 2]. Thus the scarcity of effective and commercially available NIR labels has reduced the bio-applications to few known dyes as Indocyanine Green [3, 4], which has some drawbacks as low photostability [5], metal containing fluorophores [6] or dyes with shorter emission wavelength [7]. Moreover, NIR dyes for biological applications need to be unaffected by pH variation, which can occur during the bioconjugation. It is important too, that the position of the absorption and emission bands remains as unchanged as possible in presence of biomolecules, so that the spectroscopic characterization of the labelled samples can be simply set up considering the free dyes specifications.

In this work we present the design, synthesis and characterization of novel heptamethine cyanine dyes (HCDs, in Fig. 1) with two sulfonic groups, which assure high solubility in water. Three families of dyes can be developed by a nucleophilic substitution of a basic cyanine (HCDCl, in Fig. 1) thus originating oxygen, sulphur and nitrogen

Fig. 1 The structure of HCDs



HCDCl : X=Cl; **HCDS**: X=S-Ph-NHCO(CH2)3COOH;
HCDO: X=O-Ph-NHCO(CH2)3COOH; **HCDN**: =NH(CH2)3COOH.

containing dyes [8]. The presence of heteroatoms on the γ position of the polymethine chain induces a noticeable shift in the absorption and emission spectra [8] and, in the case of nitrogen, a most important broadening of the absorption band [9]. In this work we observed that in γ-nitrogen containing dyes (HCDN) such broadening is accompanied by an increase of the Stokes' shift larger than 100 nm, to be compared with that of about 20 nm for conventional cyanine dyes. Patonay et al. reported the synthesis and characterization of a similar HCD containing nitrogen [10, 11], bearing a crown ether. In that work the nitrogen atom is directly linked to an aromatic ring which does not induce any increase of the Stokes' shift. Moreover, a discrepant discussion of the pH effect upon this dye is given. In this work we report the absorption and emission spectra of HCDN in different pH conditions, showing that the pH effect is negligible since it does not shift the absorption and emission maxima and poorly affects the emission intensity.

In order to test the efficiency of the NHS esters of HCDs dyes, a series of coupling reactions to IgG was performed and a spectroscopic analysis of the conjugated products was realized and compared to the free dyes.

Experimental

All chemicals and solvents were purchased from Sigma-Aldrich (Milan, Italy) and used as received, unless otherwise stated. Goat IgG against Microcystin were generously supplied by Abkem Iberia. HPLC analysis were performed on a Shimadzu 10 AVP (equipped with a UV-Vis detector). H^1-NMR spectra were performed on a Bruker Avance 600 using d_6-DMSO with TMS as the internal standard. NMR signals are described by use of *s* for singlet, *d* for doublet, *t* for triplet, *m* for multiplet and are expressed in δ. Mass spectrometry data were collected by a MALDI-TOF Bruker Instrument. Absorption (UV-Vis-NIR) and fluorescence spectroscopies were performed respectively by a UV-Vis-NIR-3101PC Shimadzu and by a Perkin-Elmer LS50 spectrofluorimeter equipped with a Hamamatsu R3896 photomultiplier tube (185–900 nm spectral response).

Synthesis

HCDs were synthesized by modification of the procedure described in literature [8, 12].

HCDN was synthesized through a substitution reaction of the nucleofugal Cl in the γ position of the parent dye HCDCl as follow: HCDCl (2 g, 2.88 mmol) and 6-aminohexanoic acid (3.78 g, 28.8 mmol) in anhydrous DMF (80 ml) were mixed under nitrogen atmosphere, and heated at 50°C for 34 h, then cooled down to room temperature while reacting for other 4 h. The reaction progress was monitored by the variation of Vis-NIR absorption in solutions diluted with MeOH. As the reaction proceeds the absorption band for HCDCl at 785 nm disappears, substituted by a new peak at 620 nm. The mixture was diluted in diethyl ether (200 ml) and the precipitate filtered off and dried. The crude product was dissolved in water/acetonitrile and chromatographed by MPLC on C18-RP phase using a 0 to 30% gradient of acetonitrile in water. The yield was 1.3 g (55%).^{1}H NMR (DMSO-d_6): δ (ppm) 7.63 (s, 1H, H_{Ar}), 7.60 (d, 1H, CH_{chain}), 7.07 (d, 1H, H_{Ar}), 7.57 (d, 1H, H_{Ar}), 5.79 (d, 1, CH_{chain}), 4.02 (qd, 2H, $-\underline{CH_2}CH_3$), 3.73 (t, 2H, $NH\underline{CH_2}$), 2.78 (t, 2H, H_{ring}), 2.23 (m, 2H, $\underline{CH_2}COOH$), 1.78 (m, 2H, $NHCH_2\underline{CH_2}$), 1.36 (m, 2H, $NHCH_2CH_2\underline{CH_2}$), 1.63 (s, 6H, CH_3), 1.51 (m, 2H, $\underline{CH_2}CH_2COOH$), 1.57 (m, 2H, H_{ring}), 1.22 (t, 3H, $-CH_2\underline{CH_3}$). Mass MALDI: 765 (M^+) Da. Synthesis of HCDO and HCDS follows similar procedures [13].

The dyes were modified to the correspondent NHS esters *via* the established NHS/DCC method [14]. Since activation was sometimes incomplete, reverse-phase HPLC was used to determine the percentage of fluorophore in the activate ester form by integration of the absorbance signals from the UV/Vis detector. The sample was eluted through a Nova-Pack C18RP (Waters) 3.9 × 150 mm, using a gradient 25–75% of acetonitrile in water with 0.1% trifluoroacetic acid (TFA).

Table 1 Spectroscopic data of the free HCDs and of the conjugated IgG (HCDs/IgG), in PBS pH = 7.4

Name	λ_{abs} (nm)	$\Delta\lambda_{1/2abs}$ (nm)	log ε (l mol^{-1} cm^{-1})	λ_{em} (nm)	$\Delta\lambda_{1/2em}$ (nm)	Φ	Stokes shift (nm)
HCDO	769	46	5.34	788	45	0.30	17
HCDO/IgG	774	—	—	791	—	—	—
HCDS	791	70	5.34	818	47	0.39	22
HCDS/IgG	800	—	—	823	—	—	—
HCDN	625	107	5.29	755	86	0.28	116
NHCD/IgG	618	—	—	747	—	—	—

General IgG labelling procedure

Stock solutions of NHS esters of HCDs were prepared in anhydrous DMF. IgG were labelled by adding 10:1 molar excess aliquots of ester, up to a 140 fold molar excess, to a solution of 1 mg IgG in 1 ml $NaHCO_3$ buffer solution (pH = 9.6). The reaction was carried out in the dark, at room temperature, under gentle shaking for 2.5 h and stopped by adding 150 μl of $NH_2OH{\cdot}HCl$ dissolved in 0.5 M $NaHCO_3$ including 85% NaCl. Labelled antibodies were purified using PD10 columns Sephadex® GD25 (Amersham Bioscience), using a phosphate buffer saline solution (pH = 7.4) as eluent.

The dye/protein ratios (D/P) of the conjugates were determined by the absorption spectra of the labeled proteins, registered in PBS (pH = 7.4) according to the relationship:

$$D/P = (\mathrm{Abs}_{max} \times \varepsilon_{protein})/(\mathrm{Abs}_{280} - C\mathrm{Abs}_{max})\varepsilon_{dye}$$

Where Abs_{280} is the absorption of the conjugate at 280 nm; Abs_{max} is the absorption of the conjugate at the absorption maximum of the corresponding HCD; C is the correction factor ($C = 0.29$ for heptamethine cyanine dyes); $\varepsilon_{protein}$ (187.000 l mol^{-1} cm^{-1} for IgG) and ε_{dye} (see Table 1) are the molar extinction coefficients for IgG and HCDs, respectively [14].

Spectroscopy

Absorption and emission spectra of free dyes were recorded in spectroscopic grade solvents (Figs. 2 and 3). Effect of bioconjugation upon spectral properties were evaluated by recording absorption and emission spectra of free HCDs and conjugated IgG (HCDs/IgG) in PBS (phosphate buffer saline, pH = 7.4) at room temperature. Extinction coefficients of the free dyes (ε) were calculated from the slope of a Lambert-Beer plot. Fluorescence quantum yields (Φ) were determined according to Demas [15] taking Indocyanine Green (Sigma Aldrich) as reference dye [16].

The width of absorption and emission bands were estimated by the difference of wavelength values whose intensity resulted to be the half of maximum ($\Delta\lambda_{1/2}$).

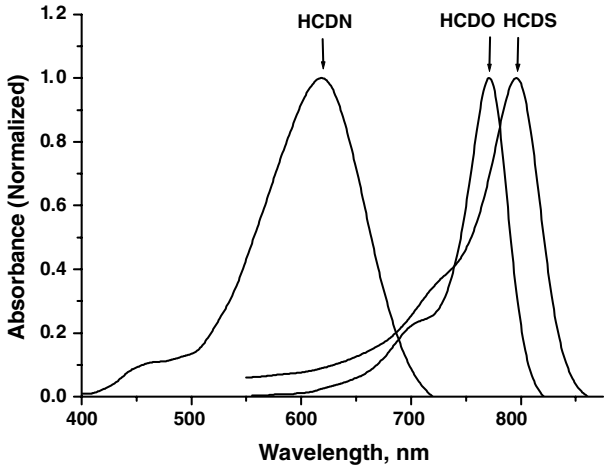


Fig. 2 Absorption spectra of HCDs (in methanol)

The effect of pH upon absorption and emission of free HCDN (Fig. 4a and b) was evaluate from solutions of equal dye concentration (1×10^{-6} M) at different pH values of phosphate buffer saline solutions (pH = 5.00; pH = 5.80; pH = 6.50; pH = 7.25; pH = 8.00; pH = 8.75; pH = 9.55).

Photostability of HDCs was measured in PBS solution (pH = 7.40), after 15 min of exposure under continuous Xenon Lamp, regulated by a monochromator at the wavelength of the absorption maximum. A performances

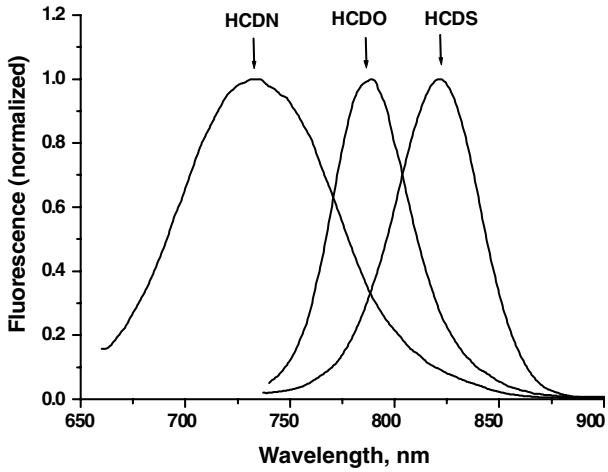


Fig. 3 Emission spectra of HCDs (in methanol)

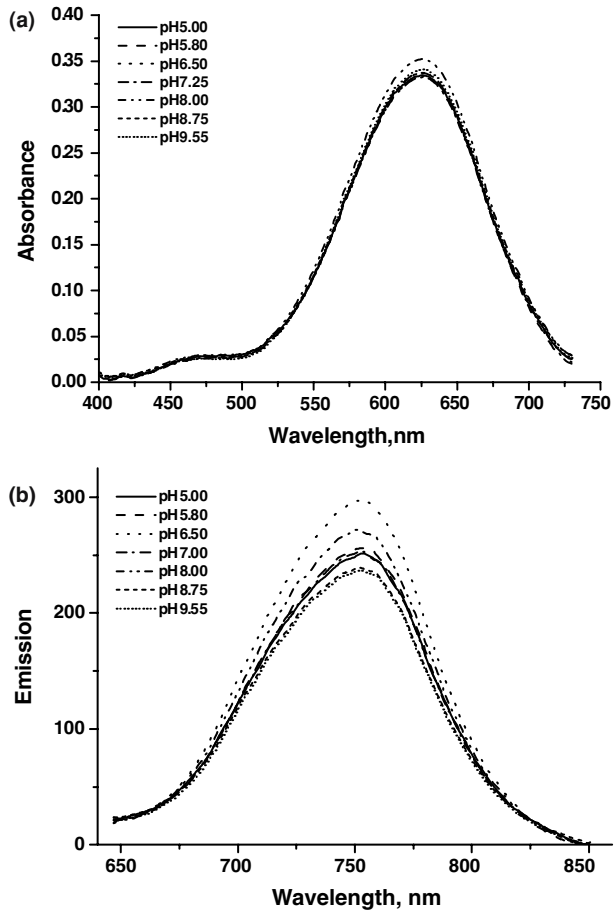


Fig. 4 pH effect upon HCDN (a) absorption, (b) emission

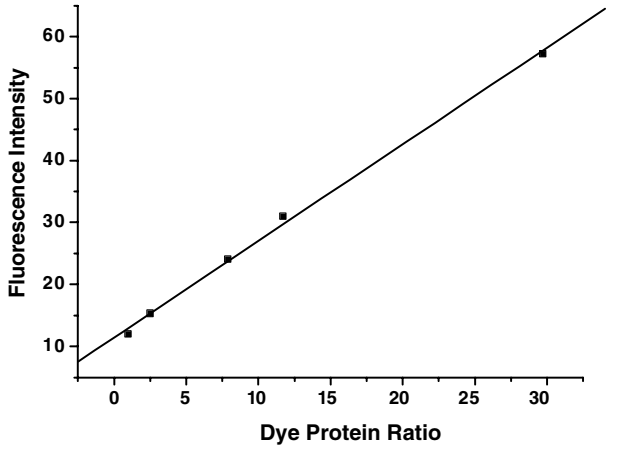


Fig. 5 Emission of IgG-HCDN with variable Dye/Protein ratio, in PBS buffer solution pH = 7.4

comparison was carried out recording the same data for Indocyanine Green (Sigma-Aldrich). The concentration for both dyes was fixed at concentration of 1×10^{-10} M, which is the normal working dilution for bioconjugation applications.

Emission intensity of increasing Dye-Protein IgG labelled with HCDN were evaluated from solutions of equal protein concentration. Fluorescence spectra of conjugated IgG were collected in PBS, pH = 7.4 (Fig. 5).

Results and discussion

Different HCDs were synthesized from the same precursor (HCDCl), which can be easily produced in large amount, through a nucleophilic substitution that follows the same synthetic procedure for each HCDs. The same simple and reproducible procedure, with high yield (>50%), was set up for the synthesis of novel near infrared emitting dyes. The purity of the compounds reaches the satisfactory value of 99% in HPLC analysis after MPLC purification. Each dye presents a spacer arm ending with a carboxylic group, which, further modified to succinimidyl ester, can react to primary amino groups of biomolecules giving a stable covalent bond. In the case of HCDO and HCDS, the heteroatoms are directly linked to an aromatic ring, while in the case of HCDN, the spacer arm is completely aliphatic. The length of the spacer arms was chosen in order to minimize the interactions between dye and protein and its effectiveness is demonstrated by the unchanged spectroscopic properties after bioconjugation. The NHS ester of HCDs are effective for bioconjugation; a 10 fold molar excess is sufficient to reach a $D/P = 1$.

HCDS and HCDO show absorption and emission bands in the NIR, with narrow profiles, as typical for cyanine dyes. With respect to the parent HCDCl, HCDS shows a bathochromic shift, HCDO an hypsochromic one, both in absorption (HCDS: +14 nm, HCDO: −7 nm) and in emission (HCDS: +9 nm, HCDO: −21 nm). On the contrary, HCDN absorption band falls at 620 nm (hypsochromic shift: −152 nm), while the emission maximum falls in the NIR at 755 nm (hypsochromic shift: −54 nm). Both absorption and emission bands are unexpectedly broad ($\Delta\lambda_{1/2} = 100$ nm) and have a surprisingly wide Stokes' shift of about 120 nm (Figs. 2 and 3). HCDs show high log ε (about 5.3) and satisfactory fluorescent quantum yield (about 0.30), as expected for heptamethine cyanine dyes (Table 1).

The peculiar photophysical properties of N-alkyl substituted heptamethine cyanine dyes are connected with the strong electron-donor ability of this group. This leads to a decrease of the effective length of the π-conjugated system. Spectroscopic studies and quantum-chemical calculations are in agreement with our data [9], while the anomalous large Stokes' shift was not previously reported. This feature can be exploited in the design of multitasking analysis with detection in the NIR. At the same time it is possible to design FRET (Fluorescence Resonance Energy Transfer) experiments in which HCDN can act as the donor, while HCDO can be the acceptor.

Table 2 Effect of the solvent upon spectroscopic features of HCDN

Solvent	λ_{abs} (nm)	$\Delta\lambda_{1/2abs}$ (nm)	log ε ($l\,mol^{-1}\,cm^{-1}$)	λ_{em} (nm)	$\Delta\lambda_{1/2em}$ (nm)	Stokes shift (nm)
Water	611	116	5.29	750	88	139
Methanol	620	108	5.39	737	86	117
Ethanol	626	108	5.42	753	88	127
2-Propanol	636	107	5.52	742	90	106
DMSO	634	123	5.25	761	87	127
DMF	636	117	5.36	761	82	125
Acetone	642	122	5.47	760	76	118

The position of the bands, for the three families of HCDs, is not significantly affected by the conjugation to IgG (Table 1). On the contrary there is a marked solvent effect upon the spectral properties of the free dyes. Solvatochromic behaviour regarding HCDN is noticeable (Table 2), both in the absorption and emission spectra. Photostability measurements show a decrease of the emission intensity of about 1% for HCDO and HCDS, of about 5% for HCDN, while the commercial Indocyanine Green, in the same conditions, shows a decrease of about 10%. The relatively high photostability can be attributed to the presence of a ring in the polymethine chain, which confers more rigidity to the *all-trans* structure and a more difficult access of the oxygen.

Absorption and emission spectra at different pH show that HCDN is not significantly affected by the change of pH (Fig. 4a and b) and the same behaviour was observed for HCDO and HCDS. The band position does not significantly change. The absorption intensity remains steady, while the fluctuation of the emission intensity are more pronounced for HCDN. However, this behaviour does not prevent the use of HCDN for biological applications.

Fluorescence measurements of the HCDN labelled IgG at different D/P ratios demonstrates that the emission increases with increasing D/P, even at high dye/protein ratios (up to $D/P = 30$), thus making this dye perfectly suitable for microscopy applications.

Conclusions

A set of novel near infrared emitting cyanine dyes, water soluble and pH unaffected have been designed and synthesized for biological applications *via* NHS ester conjugation. An increase in photostability of the dyes is registered, with respect to commercial dyes with similar features as Indocyanine Green. Among the novel dyes synthesized, HCDN shows a broad absorption band covering the visible range (400–700) and a very broad emission band centred at 755 nm with a Stokes shift of more than 100 nm, which, to our knowledge, was not previously reported. Preliminary results regarding IgG labelling with HCDs showed that interaction between dye and protein does not affect the position of the absorption and emission bands. Increasing dye/protein ratio induces a liner increase of fluorescence performances of HCDs up to 30 fold excess. The spectroscopic features observed for HCDs make them suitable for multitasking fluorescence analysis in the NIR, *in-vivo* imaging, fluorescence microscopy and fluorescence resonance energy transfer (FRET) applications.

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