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ORIGINAL PAPER

New Method for Covalent Fluorescent Biomolecule Labeling with Hemicyanine Dye

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Abstract Fluorescent chromophore, alkylamino-(tetrahydronaphthalenylidene)- benzothiazolium derivatives (HBTN dyes), are proposed as covalent labels for proteins *via* aliphatic amino groups. Spectral-luminescent properties of 3-methyl-2-{(E)-[7-(methylamino)-4,4a,5,6-tetrahydronaphthalen-2(3H)-ylidene]methyl}-1,3-benzothiazol-3-ium chloride (HBTN, $R = \mathrm{Me}$) and its predecessor, 2-[(E)-(7-methoxy-4,4a,5,6-tetrahydronaphthalen-2(3H)-ylidene)methyl]-3-methyl-1,3-benzothia-zol-3-ium chloride (ABTN), are studied for free dyes and in the presence of DNA and BSA. Considerable spectral-luminescent changes accompany the transformation of ABTN into HBTN that allows monitoring conjugation reaction. In presence of DNA and BSA the HBTN increases its emission in 15 and 4 times respectively and becomes strongly fluorescent. The conditions for labeling are developed and a model conjugate of HBTN dye with BSA is synthesized. It was shown that using of HBTN dye as a fluorescent label allows detection by eye of about 3 μg/band of BSA on polyacrylamide gel upon UV-irradiation.

Keywords Hemicyanine dyes · Protein labeling · Fluorescent detection

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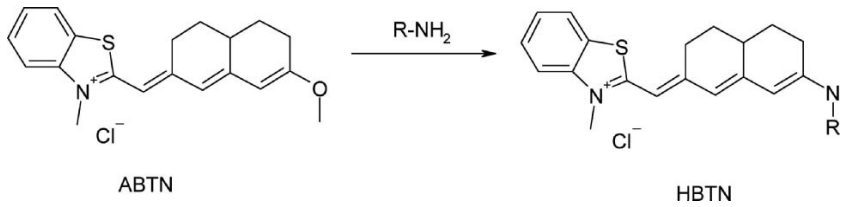
Introduction

Amino-reactive dyes are well known among the widely used labels for the modification of proteins, peptides, ligands, synthetic oligonucleotides and other biomolecules. Amine-reactive dyes are most often used to prepare bioconjugates for immunochemistry, fluorescence *in situ* hybridization (FISH), cell tracing, receptor labeling and fluorescent analog cytochemistry [1]. Commonly for the obtaining of fluorescent amino-reactive dyes the modification of the dye with isothiocyanate or *N*-hydroxysuccinimidyl ester is used [2–5].

We have reported recently a promising novel approach to oligonucleotide and peptide fluorescent labeling using pyrylium salts of cyanine dyes as alternative amine-specific reactive group. In that method low-fluorescent pyrilium dye undergoes transformation into high-fluorescent pyridinium dye upon conjugation to biomolecule [6, 7].

The aim of our work is to develop the mild conditions for the reaction of ABTN (2-[(E)-(7-methoxy-4,4a,5,6-tetrahydronaphthalen-2(3H)-ylidene)methyl]-3-methyl-1,3-benzothia-zol-3-ium chloride) with 3-aminoproionic acid, which amino group is a good model for ε-amino group of Lys. Using developed conditions the labeling of BSA with HBTN (3-methyl-2-{(E)-[7-(alkylamino)-4,4a,5,6-tetrahydronaphthalen-2(3H)-ylidene]methyl}-1,3-benzothiazol-3-ium chloride) have to be performed. Influence of the presence of biomolecules DNA and BSA (bovine serum albumin) on the fluorescent properties of both predecessor

Scheme 1 The reaction of ABTN with amines converts it into fluorescent dye HBTN



ABTN and derivative dye HBTN ($R = CH_3$, Scheme 1) would be studied.

Experimental

Materials

Anhydrous dimethylformamide (DMF) distilled under reduced pressure, ethanol and 0.05 M Tris-HCl buffer (pH 8.0) were used as solvents. Bovine serum albumin (BSA) and total deoxyribonucleic acid (DNA) from chicken erythrocytes were purchased from Sigma-Aldrich (USA). Thriethylamine and acetic acid for preparation of TEAA buffer were distilled once. Acrylamide, methylenebisacrylamide, urea, acetic acid, glycine, ascorbic acid, $FeSO_4 \times H_2O$, TEMED, potassium persulfate, Coomassie® Brilliant Blue R 250 were all purchased from Sigma-Aldrich (USA).

Synthesis

Hemicyanine dyes ABTN and methyl substituted HBTN ($R = Me$, Scheme 1) were synthesized according to [8].

Model reaction with 3-aminoproionic acid was carried out by mixing eqimolar quantities of dye solution in DMSO and the amino acid solution in 0,1 M TEAA buffer of corresponding pH (range 10–12). The reaction mixture was heated at 50 °C or left at room temperature. To monitor reaction progress VIS spectra were recorded each 30 min. from reaction mixture sample (Scheme 2).

Preparation of stock solutions

The dyes stock solutions were prepared by dissolving of the dyes in DMF, the concentration was equal to 2×10^{-3} M. Stock solutions of deoxyribonucleic acid and BSA were prepared by their dissolving in 0.05 M Tris-HCl buffer (pH 8.0). The concentrations of DNA and BSA in stock solutions were 6×10^{-3} M base pairs (b.p.) and 0.2 mg/ml respectively.

Preparation of working solutions

All working solutions were prepared immediately before the experiments. Working solution of free dyes was prepared by dilution of the dye stock solution in either buffer or commercial (96%) ethanol. Working solution of dye/DNA complex was prepared by mixing of an aliquot of the dye stock solution and an aliquot of DNA stock solution in a buffer. Working solution of dyes in presence of BSA was prepared by dilution of the dye stock solution in BSA stock solution. The concentrations of dye, DNA and BSA in working solutions were equal to 5×10^{-6} M, 6×10^{-3} M b.p. and 0.2 mg/ml respectively.

Spectroscopic measurements

Absorption spectra were recorded on a spectrophotometer Specord M40 (Carl Zeiss, Germany). Fluorescence excitation and emission spectra were taken on a fluorescence spectrophotometer Cary Eclipse (Varian, Australia). Spectroscopic measurements were performed in standard quartz cells (1×1 cm). All the measurements were carried out at room temperature.

LC-MS analysis

LC-MS experiments were carried out using chromato-mass-spectrometer Shimadzu LCMS-2010 (Shimadzu, Japan). Chromatographic separations were performed using column Discovery HS C18, 100×2 mm, 5 mkm (USA). Eluent A: 30 mg/l $AcONH_4$, 0.1% vol HCOOH, 15% vol MeOH, water, eluent B: MeOH. Gradient B0%→B80%, 7 min, flow 0.35 ml/min. Detections had been carried out consequently on diode-matrix detector SPD-M10A at 370–800 nm range and mass-selective detector LCMS-2010. Mass spectrometric investigations were carried out using electro-spray ionization (ESI) in the mode of registration of positive ions in m/z 250–1200 Da range. Data collecting and processing were performed on workstation using LabSolution® v. 2.02 software.

Protein labeling

BSA was used as a model peptide for labeling with HBTN. 500 μl of BSA solution (2 mg/ml, 1ml contains approx. 0.3 μmole of ε-aminogroups of Lys) in 0.1 M TEAA buffer (pH 11.5) was mixed with 250 μl of ABTN solution (2 mg/ml, 6 μmole/ml) in DMSO and the reaction mixture was left for a night at room temperature.

Electrophoresis

BSA labeled with HBTN was electrophoresed on acetic acid urea PAGE according to the Poperelia protocol

[9], modified by Pidura. Electrophoresis was performed in PROTEAN II xi Vertical Electrophoresis Cells (Bio-Rad, USA) at room temperature using 7% (24:1; acrylamide/methylenebisacrylamide) gels containing 8 M urea and 3% acetic acid; pH 5.5. Distilled water was used as solvent. Glycine, ascorbic acid, $FeSO_4 \times H_2O$, TEMED and potassium persulfate were added as catalysts. The running buffer (0.2% acetic acid) contained 0.2 g of glycine per liter of distilled water.

We loaded 40 μl of samples per each well in BSA concentration of 100, 60, 43, 30, 3, 0.3 and 0.03 μg/well. Samples were not boiled. Gels run at 400 volts for about 2 h at room temperature.

After electrophoresis the gels were removed from the electrophoresis apparatus and placed on a transilluminator and HBTN-BSA conjugates were visualized under UV-irradiation.

Results

Spectroscopic characterization of free dyes in ethanol and buffer

Spectral-luminescent properties of unbound ABTN and HBTN are presented in Table 1.

Absorption spectra of parent dye ABTN in ethanol have the wide low-intensive band with maxima on 495 nm and long-wave shoulder near the 535 nm (Fig. 1). When the derivative dye HBTN has the high intensive quite narrow band, short-wave shoulder on 550 nm and maxima on 584 nm are observed for this dye (Fig. 1). Despite the shapes of absorption bands for both dyes insignificantly change in aqueous buffer, intensities of the bands considerably decrease. In aqueous buffer positions of absorption spectra maxima shifted to the short-wave spectra region in 14 nm for ABTN and 4 nm in case of HBTN (Fig. 1) In the absorption spectra of ABTN aqueous solution only one band with maximum on 490 nm could be clearly distinguished. Similarly to its ethanol solution, low-intensive short-wavelength absorption spectra shoulder was observed for HBTN in buffer.

The parent dye ABTN demonstrated weak fluorescence in buffer (4 a.u.), its fluorescence excitation and emission maxima are situated on 535 nm and 556 nm correspondingly. Fluorescence intensity of derivative dye HBTN in hundred times exceeds that of the parent dye ABTN and reaches 667 a.u., that could be estimated as moderate level of emission intensity. Maxima of excitation and emission spectra for the HBTN are placed on 581 and 594 nm (Fig. 2).

Table 1 Fluorescent properties of ABTN and HBTN ($R = Me$)

	Free dye in buffer			In DNA presence		In BSA presence	
Name	λ_{ex} (nm)	λ_{em} (nm)	I_0 (a.u.)	λ_{em} (nm)	I^{DNA} (a.u.)	λ_{em} (nm)	I^{BSA} (a.u.)
ABTN	535	556	4	567	88	574	25
HBTN	581	594	665	601	9227	603	2955

λ_{ex} (λ_{em}): maximum wavelength of fluorescence excitation (emission) spectrum.

I_0 (I^{DNA}, I^{BSA}): fluorescence intensity of dye in buffer (in presence of DNA, BSA).

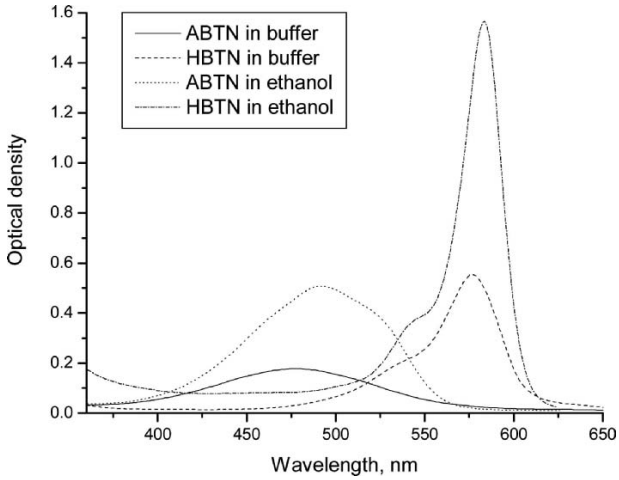


Fig. 1 Absorption spectra of 5×10^{-6} M of dyes ABTN and HBTN in ethanol and buffer

Fluorescent properties of studied dyes in the presence of DNA and BSA

Spectroscopic characteristics of studied hemicyanines in the DNA and BSA presence are described in Table 1.

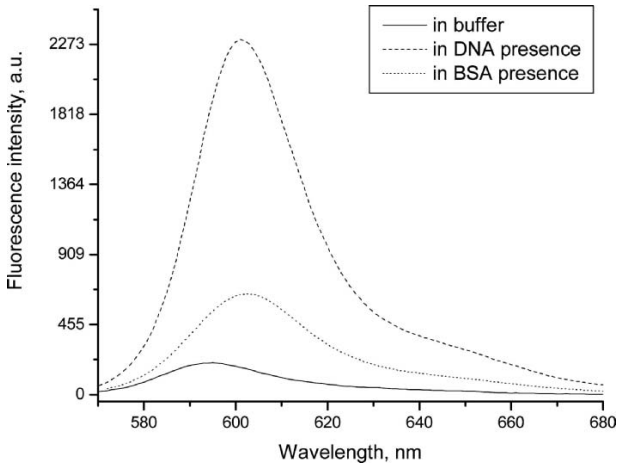


Fig. 2 Profiles of fluorescence spectra of dye HBTN ($R = CH_3$) (5×10^{-6} M) in free form and in presence of DNA (6×10^{-5} M b.p.) and BSA (0.2 mg/ml). Fluorescence was excited at 540 nm

It is necessary to point, that profiles of absorption bands and their intensities for both ABTN and HBTN in DNA or BSA presence are quite close to corresponding characteristics in buffer solutions. Both dyes ABTN and HBTN ($R = Me$) demonstrate emission increasing upon interaction with DNA. In the presence of DNA emission maximum of parent compound ABTN shifts to 567 nm, when position of fluorescence excitation maximum slightly changes (near 530 nm). Despite that this dye increases its emission up to 22 times in DNA presence, its fluorescence intensity is quite low (88 a.u.). Excitation and emission maxima of the derivative dye HBTN in the presence of DNA are slightly shifted to the long-wave region comparing with buffer solution and situated on 590 nm and 601 nm correspondingly. In DNA presence HBTN increases its emission up to 15 times and forms very "bright" fluorescent complex (emission intensity reaches 9227 a.u.). On our opinion this makes HBTN an attractive dye for the methods where using of labeled nucleotides is required.

For the studied dyes ABTN and HBTN in the presence of BSA fluorescence increasing is also observed. Excitation and emission maxima of ABTN are slightly shifted to the long-wave region and situated on 545 nm and 574 nm correspondingly. Fluorescence intensity level of ABTN in BSA presence is insignificant (25 a.u.) and emission enhancement in 5 times is observed. For the dye HBTN positions of fluorescence excitation and emission maxima in BSA presence are close to that of DNA and situated respectively on 599 nm and 603 nm. Although for this dye value of emission increasing in BSA presence (up to 4 times) is considerably lower than that for DNA, the bright fluorescence is also observed (emission intensity 2955 a.u.).

Labeling conditions and BSA labeling

ABTN is known to react readily with different nucleophiles, including amino groups. To develop the labeling conditions 3-aminoproionic acid was used as a model compound. The course of the reaction was monitored through the change of the absorption spectra of equimolar reaction mixture (Fig. 1). We considered reaction to be completed after disappearance of absorption at 500 nm.

It was found that optimum reaction conditions achieved when 0.1 M triethylammonium acetate (TEAA) buffer with pH 11-12 is used as reaction media. In this case reaction completes in 2 h at 50 °C or during one night at room temperature. The formation of the expected product HBTN ($R = (CH_2)_2COOH$) has also been proven by LC-MS analysis (Fig. 5). Main peak with m/z 381 is equal to molecular mass of the expected product, which shows that using ESI no deprotonatoin (as the molecule is already positively charged) and almost no fragmentation of dye occurs.

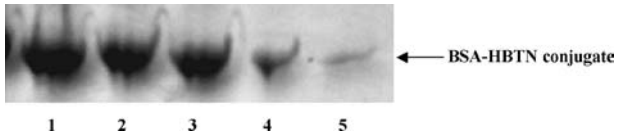


Fig. 3 Acetic acid urea polyacrylamide gel electrophoresis of BSA labeled with HBTN dye after staining with Coomassie Blue. Lanes 1–5: BSA concentration 100, 60, 43, 30, 3 μg/well

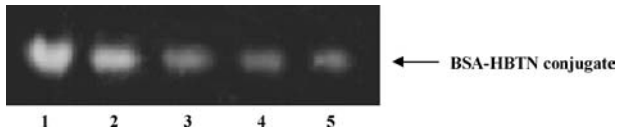


Fig. 4 Acetic acid urea polyacrylamide gel electrophoresis of BSA labeled with HBTN dye. Lanes 1–5: BSA concentration 100, 60, 43, 30, 3 μg/well. The protein bands were observed directly in gels by illumination with an ultraviolet transilluminator

BSA was used for labeling with HBTN. 10 molar excess, as for ε-aminogroups of BSA's Lys, of ABTN was used to achieve maximum degree of labeling. The reaction was carried out at room temperature to avoid denaturation of protein. After reaction was complete, the reaction mixture was directly subjected to gel electrophoresis for HBTN-BSA conjugate visualization.

The resolved during gel-electrophoresis HBTN-BSA conjugate was visualized using standard laboratory UV-transilluminator. Orange fluorescent staining of bands was observed by eye for BSA concentrations 100, 60, 43 30 and 3 μg/well (Fig. 3). The image of the gel-plate was obtained using digital camera FujiFilm S602 and glass UV-filter (that cuts off excitation irradiation but is transparent to visible light).

To confirm the presence of the protein in the fluorescently stained band, the gel plate was next stained with Coomassie Blue [10]. Using of this procedure allows us to demonstrate BSA presence for all studied concentrations (Fig. 3). Thus detectable fluorescent staining of the protein containing spots confirms the successful labeling. So it was shown that using of HBTN dye as a fluorescent label allows detection by eye of about 3 μg/band of BSA upon UV-irradiation.

Conclusions

1. New method for covalent fluorescent protein labeling with hemicyanine dyes is proposed as a simple and convenient procedure that does not require preparation of any active intermediates. As well considerable spectral-luminescent changes accompany the transformation of predecessor ABTN into derivative HBTN dye and this allows monitoring conjugation.
2. In opposite to weakly fluorescent ABTN, HBTN dye demonstrates moderate intrinsic emission intensity and forms highly fluorescent complexes with DNA or BSA.

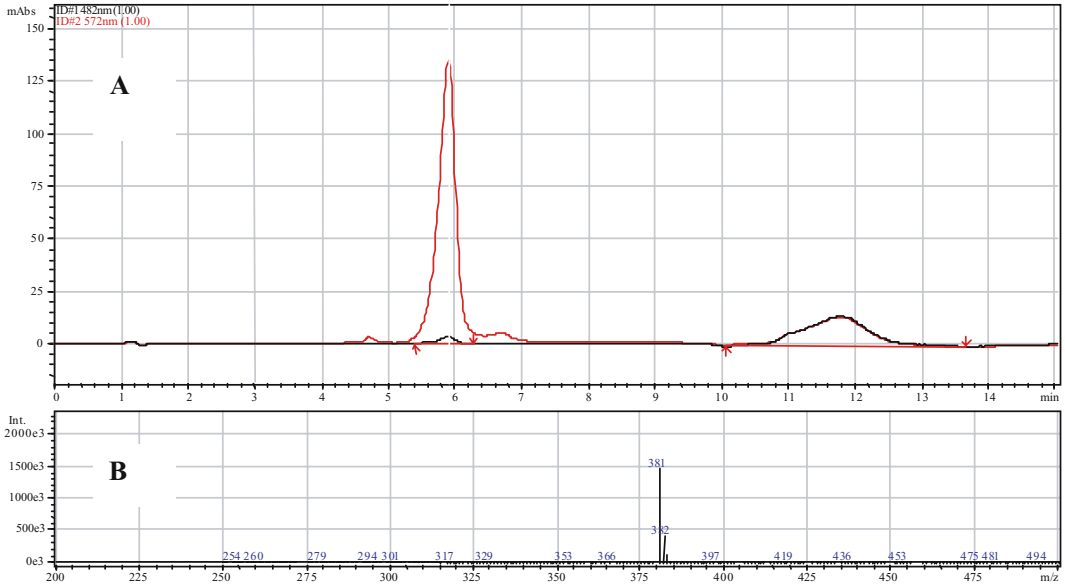


Fig. 5 LC profile (A) and MS data (B) of synthesized HBTN derivative ($R = (CH_2)_2COOH$). Desired product has a retention time approx. 6 min (A) and molecular ion with m/z 381 (B)

This makes HBTN an attractive fluorescent dye for using in techniques that require the labeling of biomolecules.

3. The conditions for labeling are developed and a conjugate of HBTN dye with BSA is obtained. It was shown that using of HBTN dye as a fluorescent label allows detection by eye of about 3 μg/band of BSA on polyacrylamide gel upon UV-irradiation.

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