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ORIGINAL PAPER

Specificity of Cyanine Dye L-21 Aggregation in Solutions with Nucleic Acids

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Abstract Optical spectroscopy experiments were used to study the features of cyanine dye 3,3′-dimethyl-9-(2-thienyl)-thiacarbocyanine iodide (L-21) aggregation in binary solutions DMF:Tris–HCl buffer (pH=8) containing nucleic acids (DNA or RNA). The appearance of absorption and luminescence bands associated with J-aggregates and dimers that are formed within the minor groove of DNA has been observed. The model of L-21 J-aggregate structure is proposed. It has been found that dimers are the building blocks of L-21 J-aggregates. Disorientation in dimers caused by the minor groove curvature is reason of observation of Davydov splitting in absorption spectrum of L-21 J-aggregates. In the solution containing DNA the absorption and luminescence bands of L-21 J-aggregates exhibit the specific properties that allows the dye L-21 to be used as a fluorescent probe for DNA detection.

Keywords Nucleic acids · Minor groove binding · J-aggregates · Cyanine dye · Fluorescence labels

Introduction

Various chemical structures of different nature such as proteins, synthetic organic molecules, fluorescent dyes and ions interact with NA and interfere with NA functions [1]. Cyanine dyes are very popular as fluorescent probes for DNA and RNA detection due to their extraordinary increase in fluorescence intensity upon binding to NA as a result of rigid fixation of trans-conformation [1–9]. There are two important ways in which cyanines can reversibly bind to DNA: by intercalation between the base pairs [1, 10–12] or by binding in the minor groove [1, 6, 7, 13]. Intercalation is inserting and stacking of molecules between the base pairs of the DNA duplex due to hydrophobic and van der Waals interaction. Intercalation is a noncovalent interaction in which the molecule is held perpendicular to the helix axis. As a rule, intercalating cyanine dyes do not exhibit specificity to DNA sequences and reveal weak GC-specificity. Also disadvantages of intercalating cyanine dyes are that they elongate the DNA helix and that they are fluorescent both in single stranded DNA and double stranded DNA [1, 10–12].

At groove binding in addition to hydrophobic and van der Waals interaction, electrostatic interaction between a dye molecule and phosphate ions takes place as well as hydrogen bonds formation with the base pairs and hydroxyl groups of sugar residua. Groove binders can be sequence specific due to the fact that they can be elongated by aggregation to extend the interactions within the groove [1, 6, 7, 13].

As a rule, monomethine dyes act as intercalators [10–12, 14], while increase in polymethine chain length leads to increasing the part of groove binders [7, 15, 16].

At groove binding, cyanine dyes can interact forming aggregates, mainly, face-to-face dimers [1, 15–17]. Dimer formation results in the appearance of a new absorption band hypsochromically shifted with respect to the monomer band. Such associates are called H-aggregates [18, 19]. For some cyanine dyes an end-to-end aggregation in minor groove of DNA was observed [16, 20–22] that results in the appearance of a new bathochromically shifted absorption

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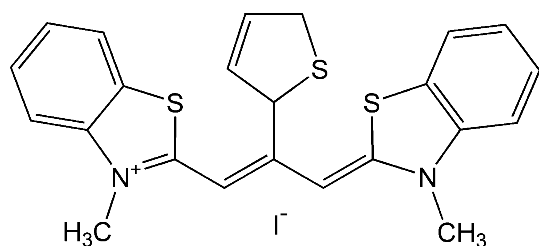

Structure 1

band [23, 24]. This type associates are called J-aggregates [23, 24]. J-aggregates formed in the minor groove can consist of several tens of monomer molecules [16, 20–22]. As geometric structure and, consequently, optical (excitonic) properties of J-aggregates are sensitive to the environment [23, 24], they could be used as probes sensitive to nucleic acids structure. For instance, due to unique properties of J-aggregates they have been used in biology as fluorescence probes for mitochondrial membrane potential monitoring [25].

In the present study, we have examined the features of J-aggregates formation for 3,3′-dimethyl-9-(2-thienyl)-thiacarbocyanine iodide (L-21) (Structure 1) in aqueous solutions containing NA.

Experimental

Cyanine dye L-21 was synthesized in Institute for Molecular Biology and Genetics (Kyiv, Ukraine). Samples for investigations were prepared from a stock solution of the dye in DMF (2 mM) by its dilution with a water buffer Tris–HCl (C=0.05 M, pH=8) at the ratio 1:19. Thus the dye concentration in the samples was equal to 10^{-4} M. DNA sodium salt (salmon testes) was dissolved in a Tris–HCl water buffer and its concentration was determined by molar absorptivity (ε=13,200 M^{-1} cm^{-1} at 260 nm, where M=mole base pairs/l). DNA concentration in all samples was 0.6×10^{-4} mole base pairs/l. Total yeast RNA was also dissolved in a Tris–HCl water buffer (4 mg/ml stock solution). For sample preparation, the stock solution was 100 times diluted. RNA concentration in all samples was 1.2×10^{-4} mole bases/l.

Luminescence and luminescence excitation spectra were recorded using a spectrofluorimeter on the base of two grating monochromators and a xenon lamp. One of the monochromators was used to select the excitation wavelength (FWHM ~20 cm^{-1}), the other one was used to record the absorption and luminescence spectra. For UV–Vis absorption spectra registration the spectrofluorimeter was supplied with incandescent and deuterium lamps. For temperature measurements, a cuvette with a sample was places in a thermostat, in which sample temperature can be smoothly changed within the 20–95 °C with a 1 °C step. Spectra were collected after no change in the absorbance.

Results and discussion

Absorption and luminescence spectra of L-21 dye in dimethylformamide (DMF) are typical for cyanine dyes (Fig. 1) with absorption maximum at 576 nm (monomer band) and luminescence maximum at 605 nm, respectively. In a binary solution DMF:Tris–HCl buffer (pH=8) the absorption spectrum changes (Fig. 1): the monomer band shifts towards short wavelengths (λ_{max}=568 nm) that could be associated with increasing solvent polarity and a new weakly intense long-wavelength band with maximum at 679 nm is revealed. The new band is broad enough ($\Delta\nu_{FWHM}$=750 cm^{-1}). The increase of short-wavelength shoulder intensity at 532 nm in the monomer absorption band with respect to the absorption spectrum in DMF (Fig. 1) should be also noted. Luminescence spectrum of L-21 in a binary solution DMF:Tris–HCl buffer also exhibits the appearance of a new very intensive band with maximum at 681 nm. The appearance of the long-wavelength absorption band and the intensive resonance luminescence one in the binary solution can be ascribed to the formation of J-aggregates of L-21 dye [23, 24]. The absorption and luminescence bands of L-21 J-aggregates observed in a binary solution DMF:Tris–HCl buffer are broad enough that could be associated with a wide length dispersion of J-aggregate chains [23, 24].

Absorption and luminescence spectra of L-21 in a binary solution DMF:Tris–HCl buffer containing NA (Figs. 2 and 3) are found to be quite different as compared to the spectra in a solution without NA (Fig. 1) that points to the interaction between the dye and NA leading to dye

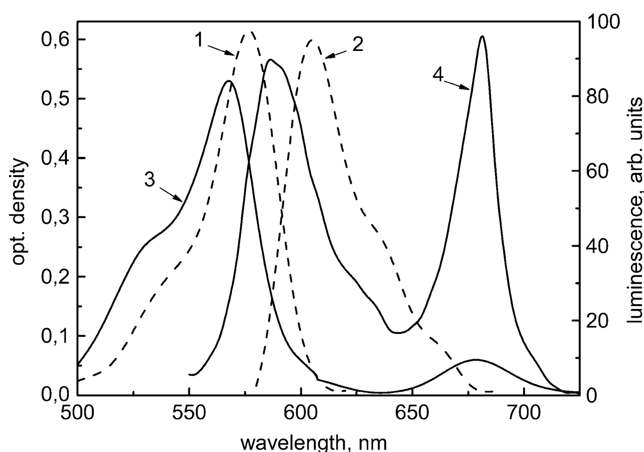

Fig. 1 Absorption (*1,3*) and luminescence (*2,4*; λ_{exc}=530 nm) spectra of L-21 in DMF (*1,2*) and in a binary solution DMF:Tris–HCl buffer (pH=8) (*3,4*)

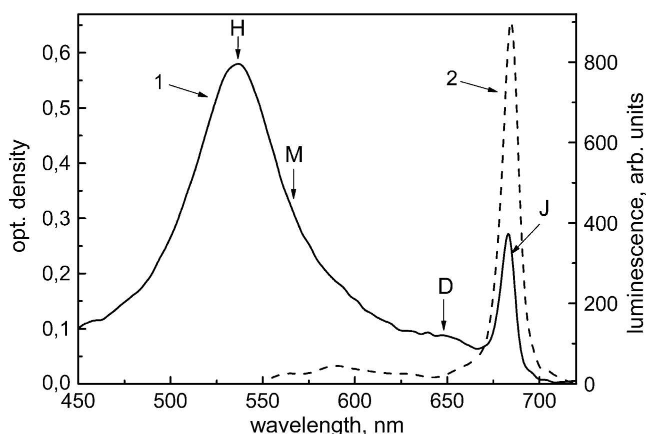

Fig. 2 Absorption (*1*) and luminescence (*2*; λ_{exc}=530 nm) spectra of L-21 (0.1 mM) in a binary solution DMF:Tris–HCl buffer with DNA

aggregation. In the absorption spectrum of L-21 in the solution with DNA (Fig. 2, curve 1) three bands can be distinguished that is ascribed to the dye aggregation. At the same time, the intensity of the monomer band (M, λ_{max}= 568 nm) is so weak that it only could be observed as a shoulder indicating the high association degree of the dye at the DNA surface. The first absorption band with the maximum at 536 nm (let's designate it as H-band) is wide and very intense band blue-shifted with respect to the monomer band. This band can be associated with aggregates of face-to-face molecular packing, so called H-aggregates [23, 24]. The second band at 683 nm maximum (let's designate it as J-band) is narrow ($\Delta\nu_{FWHM}$=150 cm^{-1}) and not very intense band shifted to the long-wavelengths. Similarly to a binary DMF:Tris–HCl buffer solution without NA, this band can be ascribed to J-aggregates (end-to-end molecular packing). The third one at 645 nm (let's designate it as D-band) is a weakly intense shoulder red-shifted with respect to the monomer band (λ_{max}= 568 nm). This band is likely to belong also to aggregates with end-to-end molecular packing, but not so extensive as J-aggregates forming J-band. At the same time, the luminescence spectrum of L-21 (at the H-band excitation) in the binary solution with DNA consists of a narrow intense luminescence band of J-aggregates ($\Delta\nu_{FWHM}$= 200 cm^{-1}, λ_{max}=685 nm) and weakly intense luminescence band of the monomers.

To determine the way by which dye molecules bind to DNA (intercalation between the base pairs or binding in the minor groove), the melting temperature for DNA containing L-21 dye and without it was measured. It has been found out that the melting point is independent of dye presence or absence and is equal to 76 °C in both cases that points to the dye binding in the minor groove of DNA [26, 27]. So, by analogy with [15, 16, 20–22], we can conclude that L-21 forms J-aggregates within the minor groove of DNA.

We can estimate exciton delocalization length for J-aggregates formed on DNA surfaces by using the equation [28]:

$$N_{del} = \frac{3}{2}\left(\frac{\Delta\nu_{mon}}{\Delta\nu_J}\right)^2 - 1 \qquad (1)$$

where $\Delta\nu_{mon}$ is the full width at half maximum (FWHM) of the monomer absorption band, $\Delta\nu_J$ is the FWHM of the J-band. Taking $\Delta\nu_{mon}$=700 cm^{-1} (from absorption spectrum of L-21 in DMF after subtraction of vibronic band), for J-aggregates of L-21 on the surface of DNA we received N_{del}≈32 monomers that is large enough value at room temperature and indicates the high ordering degree of the J-aggregates [23, 24].

In a binary solution DMF:Tris–HCl buffer containing RNA absorption and luminescence spectra of L-21 (Fig. 3) show bands similar to those observed in the solution with DNA (Fig. 2). The absorption spectrum reveals the intensity redistribution for all bands observed in the solution with DNA (Fig. 3, curve 1). Namely, the intensities of H- and J-bands decrease, while the intensities of the monomer band and D-band increase. The luminescence spectrum (Fig. 3, curve 2) shows about ten times decrease in J-aggregate luminescence intensity as compared to that in the solution with DNA. Both absorption (J, λ_{max}= 676 nm) and luminescence (λ_{max}=678 nm) band maxima of J-aggregates are slightly blue-shifted (about 7 nm) with regard to those in the solution with DNA that indicates higher disorder degree of J-aggregates [23, 26] formed on RNA surface. At the short-wavelength edge of the luminescence band of J-aggregates an additional band with maximum at 660 nm can be observed (Fig. 3, curve 2). At the D-band excitation (λ_{exc}=645 nm), a small redistribution of luminescence bands intensity is observed: the intensity of the additional band slightly increases, whereas the intensity of the J-aggregate luminescence band rather

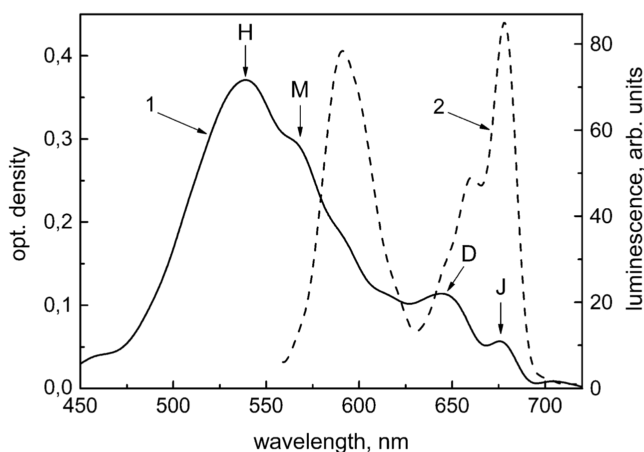

Fig. 3 Absorption (*1*) and luminescence (*2*; λ_{exc}=530 nm) spectra of L-21 (0.1 mM) in a binary solution DMF:Tris–HCl buffer with RNA

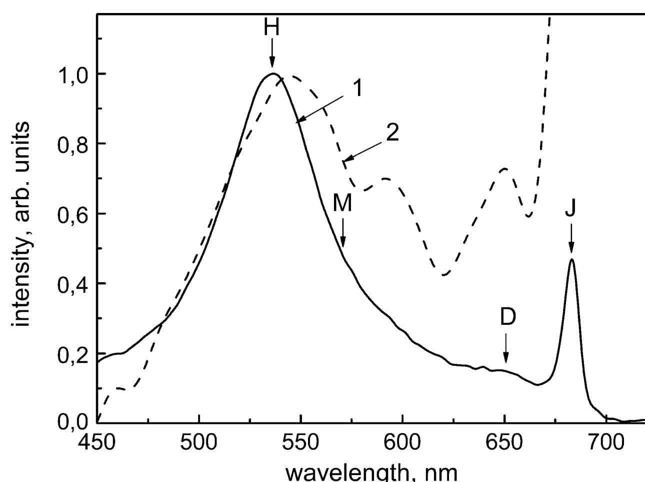

Fig. 4 Absorption (*1*) and luminescence excitation (*2*; λ_{reg}=685 nm) spectra of L-21 (0.1 mM) in a binary solution DMF:Tris–HCl buffer with DNA

decreases. That indicates that the additional luminescence band is associated with absorption of aggregates which form D-band. These aggregates are likely to be the part of extended J-aggregates.

To find out the origin of aggregation bands in the absorption spectrum (whether the bands belong to aggregates of different types or to one-type aggregates with complex structure) the luminescence excitation spectrum of L-21 J-aggregates in a binary solution DMF:Tris–HCl buffer containing DNA was recorded (Fig. 4). The comparison of the absorption and luminescence excitation spectra of L-21 J-aggregates has revealed that J-aggregate luminescence can be effectively excited in both H- and D-bands, so we can conclude that both bands can be ascribed to J-aggregates and they seem to be a result of Davydov splitting of energy levels [29]. The appearance of three bands (H-, D- and J-bands) in the spectra can be associated with existence of three structural motives in J-aggregates. The structure of the molecular chain forming a J-aggregate is clear enough to explain the appearance of H- and J-bands. Thiacarbocyanine dyes meso-substituted in 9-position are known to form dimers in such a way that the substitutes in 9-position are arranged in opposite sides [30–32]. Such a molecular arrangement means that the unit cell of the J-aggregate chain consists of two molecules that leads to Davydov splitting of energy levels [29] and in the absorption spectrum two bands (H- and J-bands) can be observed. Such an effect was observed in [33] for 9-methylthiacarbocyanine on the surface of AgHal microcrystals and for J-aggregates THIATS in a solution [34]. Really, molecules of L-21 dye are larger (~16 Å) [30–32] than the width of the minor groove (~12 Å) [1], so they are forced to settle along the minor groove. As it was shown in [15, 16] the minor groove of the DNA can accommodate only two dye molecules simultaneously as a face-to-face dimer. Further face-to-face stacking is blocked by walls of the minor groove that provokes the aggregate propagation in the end-to-end dimension. So, the H-band can be ascribed to the face-to-face dimers, while J-band is associated with extended dye aggregates consisting of dimers aligned in an end-to-end fashion within the minor groove. The main question is the origin of the D-band observed in the absorption spectra of L-21 in a binary solution with DNA and RNA (Figs. 2 and 3).

In [20, 21] for Cyanβ iPr J-aggregates a similar band was ascribed to Davydov splitting of excited electronic states of J-aggregates due to a helical structure of aggregates [18, 35] formed in the minor groove of DNA. However in [16] for $DiSC_{3+}(5)$ J-aggregates such a band was not observed.

To explore the nature of the D-band, the absorption spectra of L-21 in a binary solution DMF:Tris–HCl buffer containing DNA have been analyzed at different temperature (Fig. 5). Figure 5 illustrates that increasing temperature causes the redistribution of H-, D- and J-band intensities. H- and J-band synchronously decrease, while D-band increases. At 70 °C the H- and J-band disappear, while the intensity of D-band was a maximum. Further temperature increasing results in D-band intensity decrease and at temperature higher than DNA melting temperature (76 °C)

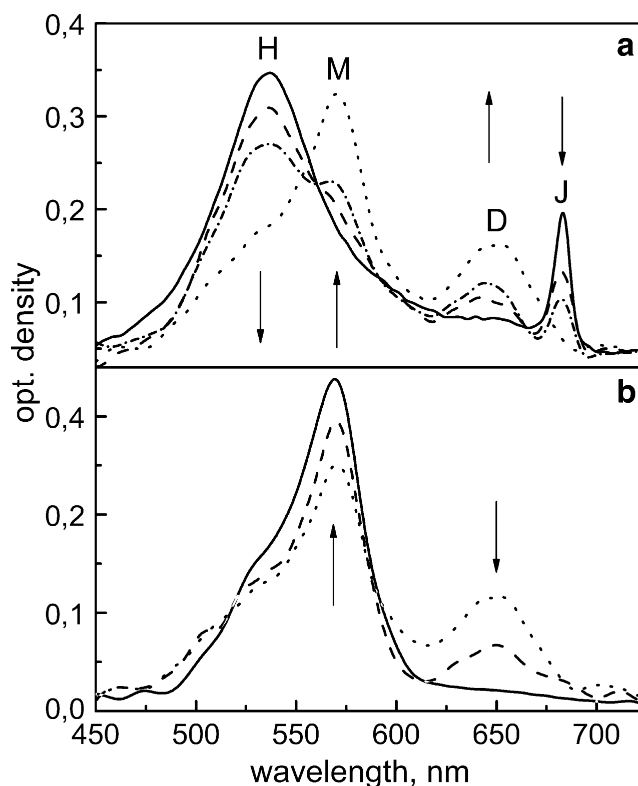

Fig. 5 Absorption spectra of L-21 in a binary solution DMF:Tris–HCl buffer with DNA at different temperatures: **a** *solid line*, 22 °C; *dashed line*, 40 °C; *dashed-dotted line*, 50 °C; *dotted line*, 70 °C; **b** *dotted line*, 70 °C; *dashed line*, 75 °C; *solid line*, 80 °C

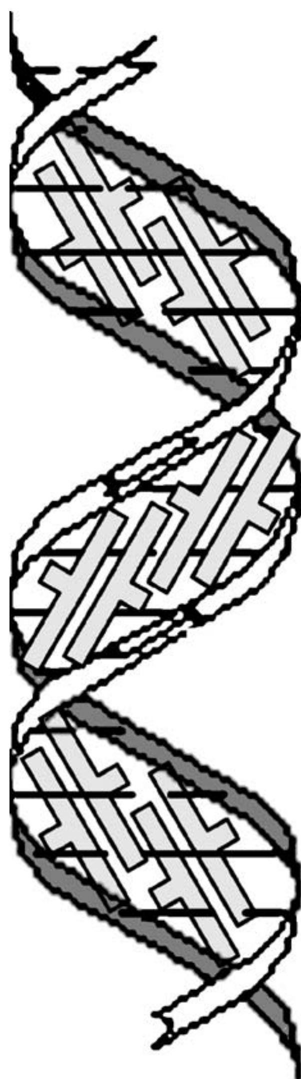

Fig. 6 Model of L-21 J-aggregate structure in minor groove of DNA. The dye molecules are shown as *rectangles*, with the 9-(2-thienyl) substituent depicted as a *flag* attached to the rectangle

it disappears. The same feature is observed for the solution containing RNA. Such a behavior of D-band and the fact that its intensity is larger in the absorption spectra of L-21 dye in a solution with RNA, where J-band is weakly intense, indicate that this band can be associated with J-aggregates with lower association degree [23, 24], which are "building blocks" of more extended J-aggregates (for example, J-dimers by analogy with H-dimers). As it was shown above, such "building blocks" in a case of L-21 dye is dimer.

In the case of dipole–dipole interaction, the shift of a dimer band with respect to a monomer one can be estimated using the equation [24]:

$$\Delta E = \frac{2|M|^2(\cos\alpha + 3\cos\theta_1\cos\theta_2)}{r^3} \quad (2)$$

where M is the transition dipole moment of monomers; α is the angle between transition dipole moments of the individual molecules, θ_1 and θ_2 are the angles between the line connecting the centers of the individual molecules and their transition dipole moments; r is the distance between the molecules forming the dimer.

For H-dimer α=0°, θ_1=θ_2=90°, hence $\Delta E = \frac{2|M|^2}{r^3}$. For J-dimer α=0°, θ_1=180°, θ_2=0° and $\Delta E = -\frac{4|M|^2}{r^3}$. So, in the case of J-dimers the shift of the dimer band with respect to the monomer one should be two times larger than in the case of H-dimers. In our case, for the H-band ΔE=1,150 cm^{-1}, whereas for the D-band ΔE=2,175 cm^{-1}. So, we can conclude that the D-band is associated with J-dimers of L-21 dye.

It could be assumed that the D-band is a result of mutual displacement of molecules forming face-to-face dimers. Due to its opposite arrangement, such a displacement can lead to the additional energy splitting. Since the intensity of D-band in the spectra is very weak as compared to the H-band (Figs. 2 and 3), the displacement is not large and are probably caused by the curvature of the minor groove where J-aggregates are formed (Fig. 6). Temperature dependent redistribution of the bands intensities (Fig. 5) indicates the change of the dimer structure from preferred face-to-face to end-to-end fashion. Indeed, after the samples cooling down the significant transformation of the absorption and

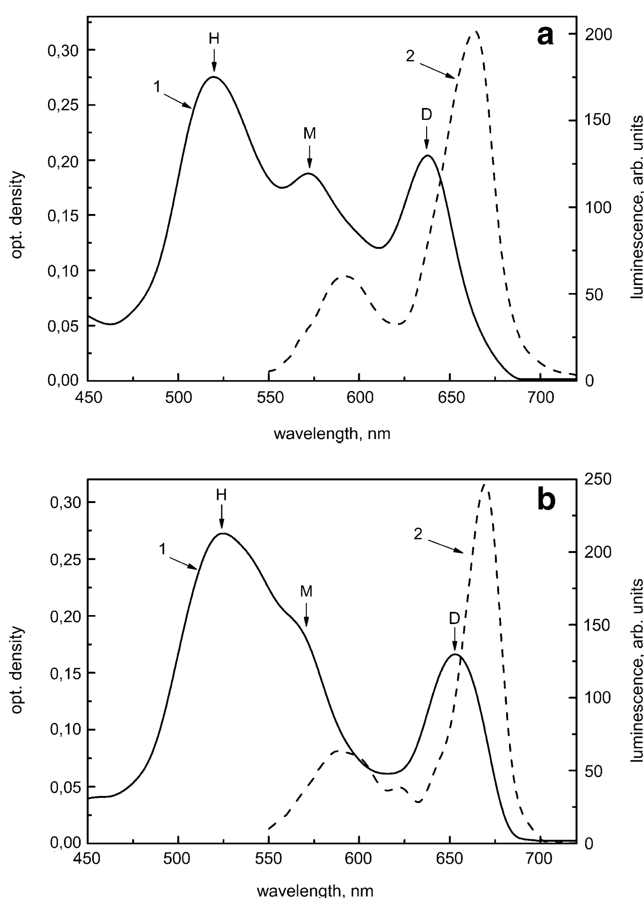

Fig. 7 Absorption (*1*) and luminescence (*2*; λ_{exc}=520 nm) spectra of L-21 (0.1 mM) in a binary solution DMF:Tris–HCl buffer with **a** DNA and **b** RNA after heating

luminescence spectra for both solutions with DNA (Fig. 7a) and RNA (Fig. 7b) is revealed. In both solutions the same features can be observed. In the absorption spectra the J-band is not manifested. The intensity of the D-band increases significantly, whereas the intensity of the H-band decreases. In the luminescence spectra the two bands are observed: the monomer band and a new band with λ_{max}=663 nm for the solution with DNA (Fig. 7a) and λ_{max}=668 nm for the solution with RNA (Fig. 7b), respectively. As a shape and a position of this new band are not changed at the excitation in the D-band, we can ascribe it to the lower sublevel of the dimer and H-band can be associated with the upper sublevel [29, 34]. Thus, the additional band revealed in the luminescence spectrum of L-21 in a solution containing RNA (Fig. 3) is really associated with the emission of dimers not included in the extended J-aggregate due to lower order degree of RNA structure as compared to DNA and, consequently, the lack of places where extended dye aggregation could take place. Let us notice that the change of L-21 J-aggregates structure under the heating is a result of the change of NA structure probably due to the formation of single-stranded DNA. This idea is proved by the fact that heating a solution containing NA without the dye and subsequent dye adding leads to the spectra similar to that presented in Fig. 7.

Conclusions

It has been shown that in a binary solution DMF:Tris–HCl buffer containing nucleic acids the dye L-21 forms extended J-aggregates which bind in minor groove of DNA. The main "building blocks" of the J-aggregates are face-to-face dimers with a slight disorientation of molecules formed dimers caused by the interaction with the walls of DNA helical minor groove. Specificity of L-21 J-aggregates formation in the solutions with DNA and RNA has been shown that is manifested in both absorption and luminescence spectra. This specificity allows this dye to be used as a fluorescent probe for NA detection.

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