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Synthesis of new benzo[*a*]phenoxazinium probes possessing carboxylic ester, hydroxyl and amino functional groups: Photophysical studies in dry ethanol and conjugation with CdTe quantum dots

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ABSTRACT

A new series of benzo[*a*]phenoxazinium chlorides possessing hydroxyl, ethyl ester and amino functional groups as terminal substituents at the 9- and 5-positions of the tetracyclic aromatic system in different combinations, was synthesized. A photophysical study was carried out in anhydrous ethanol and water, as a function of solution pH. Acid dissociation constants were estimated and found to depend on the nature of the terminal groups. Experimental evidence for an additional molecular form is presented and is compatible with J-aggregates of the deprotonated form of some of the earlier studied benzo[*a*]phenoxazinium chlorides. The benzo[*a*]phenoxazinium chloride possessing the amino terminal group was reacted with the carboxylic acid of CdTe quantum dots (QDs) to obtain a conjugate of the dye and QD. Initial photophysical characterization indicates both photoinduced electron and energy transfer between the QD and the attached benzo[*a*]phenoxazinium chloride.

1. Introduction

Synthesis of fluorescent molecules is an enthusiastic topic of interest due to their labelling ability in various analytical applications and biosciences [1–6]. In this context, the phenoxazines and benzo[*a*]phenoxazinium compounds are of prime importance due to their better photostability, high molar absorption coefficient and non-toxicity compared to other dyes [7–9]. The oxazine core in benzo[*a*]phenoxazinium compounds has its own unique synthetic conciseness and spectral properties. Owing to the synthetic and analytical importance of these heteroaromatic compounds, various researchers have reported different types of compounds with a variety of combinations [10–14].

Semiconductor nanocrystals, also called quantum dots (QDs), are an emerging new class of powerful and versatile biomedical imaging probes. Their fluorescence is unique in comparison with the former traditional organic fluorophores. QDs exhibit high quantum yields, high photostability, large absorption coefficients, continuous absorption bands with narrow and symmetric emission for multicolour capability, and many biofunctionalisation strategies. Most of the preparation techniques use a tri-*n*-octylphosphine (TOPO) based system in which the reagents are injected into a high coordinating solvent at high temperature (200–400 °C) under nitrogen or argon moisture free atmosphere [15]. Direct aqueous synthesis of semiconductor nanocrystals by employing different short-chain thiols as stabilizing agents provides a useful alternative [16]. The ability of CdTe QDs to interact with neighbouring molecules enables Förster Resonance Energy Transfer (FRET) and/or photoinduced electron transfer processes that are the basis of several sensing applications [17–19]. Recently a NADH-sensitive quantum dots were reported [20] in which the fundamental process was an electron transfer between NADH and a Nile Blue (NB) molecule attached to the QD. This process interrupted an energy transfer channel from the quantum dot to Nile Blue resulting in increased emission from the QD.

Our main research interest is focused on the design, synthesis and applications of new Nile Blue derivatives with different substituents at 5- and 9-positions [21–30]. The present work is aimed towards the synthesis of a new set of (bi)functionalised benzo[*a*]phenoxazinium chlorides possessing hydroxyl, carboxylic ester

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and amino groups as terminal of substituents at 5- and 9-positions of the heteroaromatic system, and the evaluation of its photophysical behaviour as a function of pH medium. The presence of an amino terminal group allowed the coupling of the benzo[*a*]phenoxazinium chloride with CdTe quantum dots. This conjugated system was developed with the aim of devising an efficient pH sensor which can be used for measuring the NADH levels. Furthermore, due to their relative ease of structural modifications, benzo[*a*]phenoxazinium chlorides are useful for bioconjugation.

2. Experimental section

2.1. Material and instruments

All melting points were measured on a Stuart SMP3 melting point apparatus. TLC analysis was carried out on 0.25 mm thick precoated silica plates (Merck Fertigplatten Kieselgel 60F_{254}) and spots were visualized under UV light. Chromatography on silica gel was carried out on Merck Kieselgel (230–240 mesh). IR spectra were determined on a BOMEM MB 104 spectrophotometer. UV–Vis absorption spectra (200–800 nm) were obtained using Shimadzu UV/3101PC spectrophotometer and fluorescence spectra with Spex Fluorolog spectrofluorometer. NMR spectra were obtained on a Varian Unity Plus Spectrometer at an operating frequency of 300 MHz for ^{1}H and 75.4 MHz for ^{13}C or a Bruker Avance III 400 at an operating frequency of 400 MHz for ^{1}H and 100.6 MHz for ^{13}C using the solvent peak as internal reference at 25 °C. All chemical shifts are given in ppm using δ_H Me_4Si = 0 ppm as reference and *J* values are given in Hz. Assignments were made by comparison of chemical shifts, peak multiplicities and *J* values and were supported by spin decoupling-double resonance and bidimensional heteronuclear correlation techniques. Low and high resolution mass spectrometry analysis were performed at the "C.A.C.T.I. – Unidad de Espectrometria de Masas", at University of Vigo, Spain. Commercially available reagents were used as received.

2.2. Synthetic methods for the preparation of precursors **1** and **2**

2.2.1. 3-((3-Hydroxypropyl)amino)phenol **1a**

To a solution of 3-aminophenol (0.50 g, 4.58 mmol) in ethanol (2 mL), 3-bromopropan-1-ol (0.696 g, 5.04 mmol) was added, and the resulting mixture was heated at 65 °C during 8 h. The progress of reaction was monitored by TLC (dichloromethane/methanol, 9:1). After completion of the reaction, the solvent was evaporated and the mixture was purified by column chromatography on silica using dichloromethane/methanol, mixtures of increasing polarity, as the eluent. Compound **1a** was obtained as brown oil (0.513 g, 67%). TLC (dichloromethane/methanol, 9:1): R_f = 0.56. IR (Neat): ν_{max} = 3320, 2950, 2923, 1616, 1489, 1461, 1316, 1288, 1232, 1150, 1056, 1000, 946, 859, 780, 748, 686, 665 cm^{-1}. ^{1}H NMR δ_H (CD_3OD, 400 MHz), 1.83 (2H, quin, *J* = 6.8 Hz, $NHCH_2CH_2CH_2OH$), 3.16 (2H, t, *J* = 6.8 Hz, $NHCH_2CH_2CH_2OH$), 3.69 (2H, t, *J* = 6.8 Hz, $NHCH_2CH_2CH_2OH$), 6.09–6.14 (2H, m, 2-H and 4-H), 6.16–6.20 (1H, m, 6-H), 6.92 (1H, t, *J* = 8.0 Hz, 5-H) ppm. ^{13}C NMR δ_C (CD_3OD, 100.6 MHz), 33.11 ($NHCH_2CH_2CH_2OH$), 42.03 ($NHCH_2CH_2CH_2OH$), 61.03 ($NHCH_2CH_2CH_2OH$), 100.96 (C-2), 105.25 (C-6), 106.36 (C-4), 130.73 (C-5), 151.78 (C-3), 159.24 (C-1) ppm. HRMS: *m*/*z* (EI): Found [M]$^+$: 167.0951; $C_9H_{13}NO_2$ requires [M]$^+$: 167.0946.

2.2.2. 3,3′-((3-Hydroxyphenyl)azanediyl)bis(propan-1-ol) **2a**

In the above reaction, this compound was also isolated as brown oil (0.124 g, 16%). TLC (dichloromethane/methanol, 9:1): R_f = 0.36. IR (Neat): ν_{max} = 3370, 2956, 2890, 2691, 2587, 1619, 1600, 1489, 1431, 1375, 1318, 1282, 1240, 1143, 1059, 999, 987, 942, 921, 873, 784, 695, 665 cm^{-1}. ^{1}H NMR δ_H (CD_3OD, 400 MHz), 1.77 (4H, br s, $N(CH_2CH_2CH_2OH)_2$), 3.66 (4H, t, *J* = 6.8 Hz, $N(CH_2CH_2CH_2OH)_2$), 3.77 (4H, t, *J* = 7.6 Hz, $N(CH_2CH_2CH_2OH)_2$), 7.0 (1H, d, *J* = 8.4 Hz, 6-H), 7.13–7.19 (2H, m, 2-H and 4-H), 7.43–7.49 (1H, m, 5-H) ppm. ^{13}C NMR δ_C (CD_3OD, 100.6 MHz), 28.44 ($N(CH_2CH_2CH_2OH)_2$), 58.05 ($N(CH_2CH_2CH_2OH)_2$), 59.70 ($N(CH_2CH_2CH_2OH)_2$), 110.20 (C-2), 113.91 (C-6), 118.48 (C-4), 132.56 (C-5), 139.78 (C-3), 160.00 (C-1) ppm. HRMS: *m*/*z* (EI): Found [M]$^+$: 225.1352; $C_{12}H_{19}NO_3$ requires [M]$^+$: 225.1365.

2.2.3. Ethyl 4-((3-hydroxyphenyl)amino)butanoate **1b**

To a solution of 3-aminophenol (0.436 g, 4.0 mmol) in ethanol (2 mL), ethyl 4-bromobutanoate (0.854 g, 4.4 mmol) was added, and the resulting mixture was heated at 78 °C during 6 h. The progress of reaction was monitored by TLC (dichloromethane/methanol, 9.5:0.5). After completion of the reaction, the solvent was evaporated and the mixture was purified by column chromatography on silica using dichloromethane as the eluent. Compound **1b** was obtained as brown oil (0.633 g, 71%). TLC (dichloromethane/methanol, 9.5:0.5): R_f = 0.66. IR (Neat): ν_{max} = 3398, 2981, 2938, 2873, 1715, 1655, 1616, 1600, 1515, 1499, 1475, 1446, 1415, 1374, 1343, 1301, 1236, 1184, 1160, 1106, 1028, 994, 949, 881, 830 cm^{-1}. ^{1}H NMR δ_H ($CDCl_3$, 400 MHz), 1.26 (3H, t, *J* = 6.8 Hz, OCH_2CH_3), 1.92 (2H, quin, *J* = 6.8 Hz, $NHCH_2CH_2CH_2CO_2Et$), 2.41 (2H, t, *J* = 7.2 Hz, $NHCH_2CH_2CH_2CO_2Et$), 3.12 (1H, t, *J* = 7.2 Hz, $NHCH_2CH_2CH_2CO_2Et$), 4.16 (2H, q, *J* = 7.2 Hz, OCH_2CH_3), 6.12 (1H, t, *J* = 2.4 Hz, 2-H), 6.16–6.23 (2H, m, 4-H and 6-H), 7.00 (1H, t, *J* = 8.0 Hz, 5-H). ^{13}C NMR δ_C (CD_3OD, 100.6 MHz), 14.11 (OCH_2CH_3), 24.47 ($NHCH_2CH_2CH_2CO_2Et$), 31.85 ($NHCH_2CH_2CH_2CO_2Et$), 43.30 ($NHCH_2CH_2CH_2CO_2Et$), 60.72 (OCH_2CH_3), 99.84 (C-2), 104.69 (C-6), 105.78 (C-4), 130.14 (C-5), 149.46 (C-1), 156.97 (C-3), 173.98 (CO_2Et) ppm. HRMS: *m*/*z* (EI): Found [M]$^+$: 224.12808; $C_{12}H_{18}NO_3$ requires [M]$^+$: 224.12812.

2.2.4. Diethyl 4,4′-((3-hydroxyphenyl)azanediyl)dibutanoate **2b**

In the above reaction, this compound was also isolated as brown oil (0.297 g, 22%). TLC (dichloromethane/methanol, 9.5:0.5): R_f = 0.78. IR (Neat): ν_{max} = 3417, 2980, 2939, 1731, 1714, 1621, 1575, 1504, 1463, 1372, 1300, 1165, 1097, 1030, 970, 919, 823, 756, 690, 665 cm^{-1}. ^{1}H NMR δ_H ($CDCl_3$, 400 MHz), 1.25 (6H, t, *J* = 7.2 Hz, (2 × OCH_2CH_3), 1.83–1.92 (4H, m, $N(CH_2CH_2CH_2CO_2Et)_2$), 2.32 (4H, t, *J* = 7.2 Hz, $N(CH_2CH_2CH_2CO_2Et)_2$), 3.26 (4H, t, *J* = 7.6 Hz, $N(CH_2CH_2CH_2CO_2Et)_2$), 4.14 (4H, q, *J* = 7.2 Hz, 2 × OCH_2CH_3), 6.18 (1H, dd, *J* = 7.2 and 1.2 Hz, 4-H), 6.22–6.26 (2H, m, 6-H and 2-H), 7.02 (1H, t, *J* = 8.0 Hz, 5-H). ^{13}C NMR δ_C ($CDCl_3$, 100.6 MHz), 14.00 (2 × OCH_2CH_3), 22.22 ($N(CH_2CH_2CH_2CO_2Et)_2$), 31.35 ($N(CH_2CH_2CH_2CO_2Et)_2$), 50.03 ($N(CH_2CH_2CH_2CO_2Et)_2$), 60.49 (2 × OCH_2CH_3), 99.20 (C-6), 103.20 (C-4), 104.38 (C-2), 129.92 (C-5), 149.10 (C-1), 157.24 (C-3), 173.58 (CO_2Et). HRMS: *m*/*z* (EI): Found [M]$^+$: 337.1905; $C_{18}H_{27}NO_5$ requires [M]$^+$: 337.1889.

2.3. Synthetic methods for the preparation of nitrosophenols **3** and **4**

2.3.1. 5-((3-Hydroxypropyl)amino)-2-nitrosophenol hydrochloride **3a**

To an ice-cold solution of 3-((3-hydroxypropyl)amino)phenol **1a** (0.150 g, 0.898 mmol) in ethanol (2 mL), 2M hydrochloric acid (0.36 mL) was added and stirred during 5 min. The solution of sodium nitrite (0.068 g, 0.988 mmol) in water (0.1 mL) was then added drop-wise within an interval of 15–20 min. The resulting mixture was stirred for 3 h and monitored by TLC (dichloromethane/methanol, 9:1 and 5:5). After evaporation of the reaction mixture, compound **3a** was obtained as an orange oily solid (0.177 g) and was used in the following step without any purification.

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*2.3.2. Ethyl 4-((3-hydroxy-4-nitrosophenyl)amino)butanoate hydrochloride **3b***

Starting from ethyl 4-((3-hydroxyphenyl)amino)butanoate **1b** (0.140 g, 0.627 mmol) in ethanol (1 mL), and 2M hydrochloric acid (0.24 mL), using sodium nitrite (0.047 g, 0.69 mmol) in water (0.1 mL), and following the same procedure as described before for the preparation of **3a** (TLC, dichloromethane/methanol, 9.7:0.3 and 5:5), compound **3b** was obtained as an orange oily solid (0.180 g) and was used in the following step without any purification.

*2.3.3. Diethyl 4,4′-((3-hydroxy-4-nitrosophenyl)azanediyl) dibutanoate hydrochloride **4***

Starting from diethyl 4,4′-((3-hydroxyphenyl)azanediyl)dibutanoate **2b** (0.100 g, 0.296 mmol) in ethanol (1 mL) and 2 M hydrochloric acid (0.12 mL), using sodium nitrite (0.023 g, 0.326 mmol) in water (0.1 mL), and following the same procedure as described before for the preparation of **3a**, compound **4** was obtained as an orange oily solid (0.109 g) and was used in the following step without any purification.

*2.4. General procedure for the synthesis of benzo[a]phenoxazines **6** and **7***

To a cold solution (ice bath) of nitrosophenols **3** or **4**, in ethanol (2 mL), precursors **5** (0.5 eq.) and concentrated hydrochloric acid (0.27 mL) were added. The mixture was refluxed during the time mentioned below, and monitored by TLC. After evaporation of the solvent and column chromatography purification on silica gel with dichloromethane and dichloromethane/methanol, mixtures of different polarity, as the eluent, the required dye **6** or **7** was obtained as a blue solid.

*2.4.1. 3-Hydroxy-N-(5-(propylamino)-9H-benzo[a]phenoxazin-9-ylidene)propan-1-aminium chloride **6a***

The product of the reaction of **3a** (0.138 g, 0.677 mmol) in ethanol (2 mL) and concentrated hydrochloric acid (1.9×10^{-3} mL) with *N*-propylnaphthalen-1-amine **5a** (0.0627 g, 0.338 mmol) (reflux time 8.5 h), was chromatographed with dichloromethane and dichloromethane/methanol 9.3:0.7 to give compound **6a** as a blue solid (0.059 g, 21%). mp 178.7–180.2 °C. R_f = 0.27 (dichloromethane/methanol, 9:1). FTIR (KBr 1%): ν_{max} = 3389, 2961, 2928, 1637, 1590, 1549, 1519, 1460, 1435, 1384, 1314, 1289, 1258, 1181, 1127, 1062, 1005, 874, 832, 772, 738, 703 cm^{-1}. ^1H NMR δ_H (CD_3OD, 400 MHz), 1.14 (3H, t, *J* = 7.2 Hz, $NHCH_2CH_2CH_3$), 1.88–1.99 (4H, m, $NHCH_2CH_2CH_3$ and $NHCH_2CH_2CH_2OH$), 3.52 (2H, t, *J* = 6.8 Hz, $NHCH_2CH_2CH_3$), 3.70 (2H, t, *J* = 6.8 Hz, $NHCH_2CH_2CH_2OH$), 3.77 (2H, t, *J* = 6.0 Hz, $NHCH_2CH_2CH_2OH$), 6.80 (1H, s, 8-H), 6.92 (1H, s, 6-H), 7.01 (1H, d, *J* = 5.6 Hz, 10-H), 7.74 (1H, br s, 11-H), 7.78 (1H, t, *J* = 7.6 Hz, 3-H), 7.87 (1H, t, *J* = 7.2 Hz, 2-H), 8.31 (1H, d, *J* = 8.0 Hz, 4-H), 8.68 (1H, d, *J* = 8.0 Hz, 1-H) ppm. ^{13}C NMR δ_C (CD_3OD, 100.6 MHz), 11.73 ($NHCH_2CH_2CH_3$), 23.07 ($NHCH_2CH_2CH_3$), 31.92 ($NHCH_2CH_2CH_2OH$), 42.64 ($NHCH_2CH_2CH_3$), 47.73 ($NHCH_2CH_2CH_2OH$), 60.78 ($NHCH_2CH_2CH_2OH$), 95.24 (C-6), 95.97 (C-8), 122.01 (Ar–C), 124.20 (C-4), 124.96 (C-10), 125.70 (Ar–C), 125.84 (C-1), 129.61 (Ar–C), 131.18 (C-11), 131.35 (C-3), 133.43 (Ar–C), 134.54 (C-2), 148.14 (Ar–C), 151.88 (C-5), 153.24 (Ar–C), 160.31 (C-9) ppm. HRMS: *m*/*z* (EI): Found $[M+1]^+$: 362.18706; $C_{22}H_{24}N_3O_2$ requires $[M+1]^+$: 337.1889.

*2.4.2. N-(5-((3-aminopropylamino)-9H-benzo[a]phenoxazin-9-ylidene)-3-hydroxypropane-1-aminium chloride hydrobromide **6b***

The product of the reaction of **3a** (0.177 g, 0.898 mmol) in ethanol (2 mL) and concentrated hydrochloric acid (2.4×10^{-3} mL) with N^1-(naphthalen-2-yl)propane-1,3-diamine hydrobromide **5b** (0.126 g, 0.449 mmol) (reflux time 13 h), was chromatographed with dichloromethane and dichloromethane/methanol 8.0:2.0 to give compound **6b** as a blue solid (0.235 g, 53%). mp > 300 °C. R_f = 0.10 (dichloromethane/methanol, 9:1). IR (KBr 1%) ν_{max} = 3233, 1638, 1589, 1547, 1524, 1496, 1454, 1434, 1326, 1155, 1123, 1058, 1009, 949, 876, 831, 778, 757, 743, 705 cm^{-1}. ^1H NMR δ_H (CD_3OD, 400 MHz), 1.94–2.00 (2H, m, $NHCH_2CH_2CH_2OH$), 2.23–2.31 (2H, m, $NHCH_2CH_2CH_2NH_2.HBr$), 3.20 (2H, t, *J* = 7.2 Hz, $NHCH_2CH_2CH_2NH_2.HBr$), 3.52 (2H, t, *J* = 7.2 Hz, $NHCH_2CH_2CH_2NH_2.HBr$), 3.75 (2H, t, *J* = 6.0 Hz, $NHCH_2CH_2CH_2OH$), 3.88 (2H, t, *J* = 7.2 Hz, $NHCH_2CH_2CH_2OH$), 6.81 (1H, s, 8-H), 7.03 (1H, s, 6-H), 7.15 (1H, d, *J* = 9.6 Hz, 10-H), 7.78 (1H, d, *J* = 9.2 Hz, 11-H), 7.81 (1H, t, *J* = 7.2 Hz, 3-H), 7.91 (1H, t, *J* = 8.0 Hz, 2-H), 8.47 (1H, d, *J* = 8.0 Hz, 4-H), 8.61 (1H, d, *J* = 8.4 Hz, 1-H) ppm. ^{13}C NMR δ_C (CD_3OD, 100.6 MHz), 27.60 ($NHCH_2CH_2CH_2NH_2.HBr$), 32.52 ($NHCH_2CH_2CH_2OH$), 38.43 ($NHCH_2CH_2CH_2NH_2.HBr$), 41.68 ($NHCH_2CH_2CH_2NH_2.HBr$), 42.48 ($NHCH_2CH_2CH_2OH$), 60.09 ($NHCH_2CH_2CH_2OH$), 94.31 (C-8), 95.41 (C-6), 120.53 (Ar–C), 124.16 (C-4), 124.66 (C-10), 125.45 (C-1), 125.97 (Ar–C), 129.53 (Ar–C), 130.82 (C-3), 132.77 (Ar–C), 132.88 (C-2), 134.08 (C-11), 147.21 (Ar–C), 149.10 (Ar–C), 158.50 (C-5), 159.0 (C-9) ppm. HRMS: *m*/*z* (EI): Found $[M+1]^+$: 377.19680; $C_{22}H_{25}N_4O_2$ requires $[M+1]^+$: 377.19720.

*2.4.3. 4-Ethoxy-4-oxo-N-(5-(propylamino)-9H-benzo[a] phenoxazin-9-ylidene)butan-1-aminium chloride **6c***

The product of the reaction of **3b** (0.114 g, 0.448 mmol) in ethanol (3 mL) and concentrated hydrochloric acid (1.2×10^{-3} mL) with *N*-propylnaphthalen-1-amine **5a** (0.041 g, 0.22 mmol) (reflux time 8.5 h), was chromatographed with dichloromethane and dichloromethane/methanol 9.6:0.4, to give compound **6c** as a blue solid (0.121 g, 59%). mp 115.6–116.7 °C. R_f = 0.40 (dichloromethane/methanol, 9:1). IR (KBr 1%): ν_{max} = 3450, 2926, 2855, 1728, 1641, 1591, 1548, 1495, 1456, 1434, 1384, 1282, 1160, 1122, 1094, 821, 777 cm^{-1}. ^1H NMR δ_H (CD_3OD, 400 MHz), 1.13 (3H, t, *J* = 7.2 Hz, $NHCH_2CH_2CH_3$), 1.30 (3H, t, *J* = 7.2 Hz, $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 1.86–1.91 (2H, m, $NHCH_2CH_2CH_3$), 1.98–2.05 (2H, m, $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 2.55, (2H, t, *J* = 6.4 Hz, $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 3.38 (2H, t, *J* = 6.8 Hz, $NHCH_2CH_2CH_3$), 3.63 (2H, t, *J* = 6.8 Hz, $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 4.20 (2H, q, *J* = 7.2 Hz, $NCH_2CH_2CH_2CO_2CH_2CH_3$), 6.63 (1H, s, 8-H), 6.76 (1H, s, 6-H), 7.0 (1H, d, *J* = 8.4 Hz, 10-H), 7.59 (1H, d, *J* = 8.8 Hz, 11-H), 7.72 (1H, t, *J* = 7.6 Hz, 3-H), 7.80 (1H, t, *J* = 7.6 Hz, 2-H), 8.22 (1H, d, *J* = 7.6 Hz, 4-H), 8.63 (1H, d, *J* = 8.4 Hz, 1-H) ppm. ^{13}C NMR δ_C (CD_3OD, 100.6 MHz), 11.78 ($NHCH_2CH_2CH_3$), 14.58 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 23.04 ($NHCH_2CH_2CH_3$), 24.94 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 32.07 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 43.80 ($NHCH_2CH_2CH_3$), 47.42 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 61.75 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 94.27 (C-8), 95.60 (C-6), 123.82 (C-4), 124.54 (C-10), 125.37 (C-1), 130.88 (C-3), 131.72 (2 × Ar–C), 132.20 (2 × Ar–C), 132.85 (C-2), 133.76 (C-11), 134.42 (2 × Ar–C), 157.83 (C-5), 159.11 (C-9), 175.21 (CO_2Et) ppm. HRMS: *m*/*z* (EI): Found $[M+1]^+$: 418.21327; $C_{25}H_{28}N_3O_3$ requires $[M+1]^+$: 418.21324.

*2.4.4. N-(5-(3-aminopropylamino)-9H-benzo[a]phenoxazin-9-ylidene-4-ethoxy-4-oxobutan-1-aminium chloride **6d***

The product of the reaction of **3b** (0.114 g, 0.448 mmol) in ethanol (3 mL) and concentrated hydrochloric acid (1.2×10^{-3} mL) with N^1-(naphthalen-2-yl)propane-1,3-diamine hydrobromide **5b** (0.063 g, 0.224 mmol) (reflux time 6 h 40 min), was chromatographed with dichloromethane and dichloromethane/methanol 9:1, to give compound **6d** as a blue solid (0.168 g, 68%). mp = 124.5–125.6 °C. R_f = 0.14 (dichloromethane/methanol, 9:1). IR (KBr 1%): ν_{max} = 3423, 3237, 1719, 1640, 1590, 1548, 1523, 1497, 1456, 1409, 1328, 1283, 1259, 1156, 1124, 1094, 1007, 843, 776 cm^{-1}. ^1H NMR δ_H (CD_3OD, 400 MHz), 1.29 (3H, t, *J* = 7.2 Hz,

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($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 1.37–1.44 (2H, m, $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 2.13 (2H, br s, $NHCH_2CH_2CH_2NH_2.HBr$), 2.37 (2H, br s, $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 2.65 (2H, t, J = 6.4 Hz, ($NHCH_2CH_2CH_2NH_2.HBr$), 3.42–3.48 (2H, m, $NHCH_2CH_2CH_2NH_2.HBr$), 3.62 (2H, t, J = 8.0 Hz, $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 3.73 (2H, q, J = 7.2 Hz, $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 6.55 (1H, s, 8-H), 6.80 (1H, s, 6-H), 7.01 (1H, d, J = 8.0 Hz, 10-H), 7.45 (1H, d, J = 7.6 Hz, 11-H), 7.70 (1H, t, J = 7.6 Hz, 3-H), 7.79 (1H, br s, 2-H), 8.28 (1H, d, J = 7.6 Hz, 4-H), 8.42 (1H, d, J = 6.8 Hz, 1-H) ppm. ^{13}C NMR δ_C (CD_3OD, 100.6 MHz), 18.32 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 24.83 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 27.55 ($NHCH_2CH_2CH_2NH_2.HBr$), 29.99 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 31.79 ($NHCH_2CH_2CH_2NH_2.HBr$), 43.90 ($NHCH_2CH_2CH_2NH_2.HBr$), 58.26 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 61.22 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 94.25 (C-8), 97.21 (C-6), 123.95 (2 × Ar–C), 124.05 (C-4), 124.94 (C-1), 130.59 (C-3), 131.55 (Ar–C), 132.33 (C-10), 132.72 (C-2), 133.31 (Ar–C), 133.66 (C-11), 151.98 (2 × Ar–C), 157.84 (C-5), 158.31 (C-9), 175.17 (CO_2Et) ppm. HRMS: m/z (EI): Found $[M+1]^+$: 433.22420; $C_{23}H_{25}N_4O_3$ requires $[M+1]^+$: 433.22417.

2.4.5. 4-Ethoxy-N-(5-((3-hydroxypropyl)amino)-9H-benzo[a] phenoxazin-9-ylidene)-4-ethoxy-4-oxobutan-1-aminium chloride **6e**

The product of the reaction of **3b** (0.114 g, 0.448 mmol) in ethanol (3 mL) and concentrated hydrochloric acid (1.2×10^{-3} mL) with 3-(naphthalen-1-ylamino)propan-1-ol **5c** (0.045 g, 0.224 mmol) (reflux time 7 h), was chromatographed with dichloromethane and dichloromethane/methanol 9:1, to give compound **6e** as a blue solid, (0.100 g, 47%). mp = 127.9–130.8 °C. R_f = 0.35 (dichloromethane/methanol, 9:1). IR (KBr 1%): ν_{max} = 3419, 2928, 1718, 1640, 1591, 1548, 1495, 1458, 1434, 1384, 1325, 1282, 1177, 1158, 1126, 1090, 826, 776 cm^{-1}. ^{1}H NMR δ_H (CD_3OD, 400 MHz), 1.30 (3H, t, J = 7.2 Hz, $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 1.98–2.02 (2H, m, $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 2.04–2.07 (2H, m, $NHCH_2CH_2CH_2OH$), 2.52 (2H, t, J = 7.2 Hz, $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 3.37 (2H, br s, $NHCH_2CH_2CH_2OH$), 3.72 (2H, t, J = 5.6 Hz, $NHCH_2CH_2CH_2OH$), 3.81 (2H, t, J = 5.6 Hz, $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 4.21 (2H, q, J = 7.2 Hz, $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 6.51 (1H, s, 8-H), 6.66 (1H, s, 6-H), 6.88 (1H, d, J = 8.0 Hz, 10-H), 7.48 (1H, d, J = 9.2 Hz, 11-H), 7.64 (1H, t, J = 7.6 Hz, 3-H), 7.73 (1H, t, J = 7.2 Hz, 2-H), 8.07 (1H, d, J = 8.0 Hz, 4-H), 8.50 (d, J = 8.0 Hz, 1H, 1-H) ppm. ^{13}C NMR δ_C (CD_3OD, 100.6 MHz), 14.60 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 24.93 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 30.80 ($NHCH_2CH_2CH_2OH$), 32.10 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 43.39 ($NHCH_2CH_2CH_2OH$), 43.83 ($NHCH_2CH_2CH_2OH$), 60.45 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 61.77 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 94.10 (C-8), 95.74 (C-6), 123.63 (2 × Ar–C), 124.31 (C-4), 125.28 (C-1), 130.57 (C-3), 130.68 (2 × Ar–C), 130.79 (C-10), 132.77 (C-2), 134.11 (C-11), 149.68 (Ar–C), 149.75 (Ar–C), 157.75 (C-5), 158.76 (C-9), 174.79 (CO_2Et) ppm. HRMS: m/z (EI): Found $[M+1]^+$: 434.20743; $C_{25}H_{28}N_3O_4$ requires $[M+1]^+$: 434.20733.

2.4.6. 4-Ethoxy-N-(5-((4-ethoxy-4-oxobutyl)amino)-9H-benzo[a] phenoxazin-9-ylidene)-4-oxobutan-1-aminium chloride **6f**

The product of the reaction of **3b** (0.114 g, 0.448 mmol) in ethanol (3 mL) and concentrated hydrochloric acid (1.2×10^{-3} mL) with ethyl 4-(naphthalen-1-ylamino)butanoate **5d** (0.058 g, 0.224 mmol) (reflux time 7 h), was chromatographed with dichloromethane and dichloromethane/methanol 9.7:0.3, to give compound **6f** as a blue solid (0.104 g, 44%). mp 112.1–114.5 °C. R_f = 0.58 (dichloromethane/methanol, 9:1). IR (KBr 1%): ν_{max} = 3433, 3225, 2984, 1727, 1641, 1590, 1546, 1495, 1455, 1435, 1383, 1326, 1282, 1183, 1158, 1127, 1090, 1032, 1007, 949, 826, 775, 745, 706 cm^{-1}. ^{1}H NMR δ_H (CD_3OD, 400 MHz), 1.30 (6H, t, J = 7.2 Hz, 2 × $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 1.96–2.03 (4H, m, 2 × $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 2.49–2.56 (4H, m, 2 × $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 3.26 (2H, br s, $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 3.52 (2H, br s, $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 4.20 (4H, q, J = 7.2 Hz, 2 × $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 6.31 (1H, s, 8-H), 6.47 (1H, s, 6-H), 6.77-6.87 (1H, m, 10-H), 7.25 (1H, d, J = 6.8 Hz, 11-H), 7.51 (1H, br s, 3-H), 7.59 (1H, t, J = 6.8 Hz, 2-H), 7.91 (1H, br s, 4-H), 8.22 (1H, d, J = 7.6 Hz, 1-H) ppm. ^{13}C NMR δ_C (CD_3OD, 100.6 MHz), 14.47 (2 × $NHCH_2CH_2CH_2CO_2CH_2CH_3$), 24.66 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 24.89 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 31.95 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 32.51 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 43.86 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 44.91 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 61.75 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 61.89 ($NHCH_2CH_2CH_2CO_2CH_2CH_3$), 93.96 (C-8), 95.13 (C-6), 123.65 (2 × Ar–C), 123.97 (C-4), 124.97 (C-1), 130.63 (C-3), 131.51 (2 × Ar–C), 131.54 (C-2), 132.62 (C-10), 133.41 (C-11), 149.32 (Ar–C), 151.84 (Ar–C), 155.52 (C-5), 158.40 (C-9), 174.82 (CO_2Et) ppm. HRMS: m/z (EI): Found $[M+1]^+$: 490.23435; $C_{28}H_{32}N_3O_5$ requires $[M+1]^+$: 490.23430.

2.4.7. 4-Ethoxy-N-(4-ethoxy-4-oxobutyl)-4-oxo-N-(5-(propylamino)-9H-benzo[a]phenoxazin-9-ylidene)butan-1-aminium chloride **7**

The product of the reaction of **4** (0.109 g, 0.297 mmol) in ethanol (2 mL) and concentrated hydrochloric acid (8.0×10^{-3} mL) with *N*-propylnaphthalen-1-amine **5a** (0.028 g, 0.148 mmol) (reflux time 9.5 h), was chromatographed with dichloromethane and dichloromethane/methanol 9.5:0.5, to give compound **7** blue solid, (0.087 g, 50%). Mp 192.3–195.1 °C. R_f = 0.67 (dichloromethane/methanol, 9:1). IR (KBr 1%): ν_{max} = 2923, 2853, 1731, 1721, 1637, 1589, 1545, 1499, 1435, 1375, 1334, 1322, 1290, 1230, 1182, 1162, 1146, 1127, 1100, 1054, 1001, 918, 807, 753, 728, 665 cm^{-1}. ^{1}H NMR δ_H (CD_3OD, 400 MHz), 1.13 (3H, t, J = 7.2 Hz, $NHCH_2CH_2CH_3$), 1.33 (6H, t, J = 7.2 Hz, $N(CH_2CH_2CH_2CO_2CH_2CH_3)_2$), 1.86–1.92 (2H, m, $NHCH_2CH_2CH_3$), 1.99–2.03 (4H, m, $N(CH_2CH_2CH_2CO_2Et)_2$), 2.53 (4H, t, J = 6.8 Hz, $N(CH_2CH_2CH_2CO_2Et)_2$), 3.59–3.66 (6H, m, $NHCH_2CH_2CH_3$ and $N(CH_2CH_2CH_2CO_2Et)_2$), 4.23 (4H, q, J = 7.2 Hz, $N(CH_2CH_2CH_2CO_2CH_2CH_3)_2$), 6.76 (1H, s, 6-H), 6.89 (1H, s, 8-H), 7.27 (1H, d, J = 8.0 Hz, 10-H), 7.62 (1H, d, J = 8.0 Hz, 11-H), 7.69 (1H, t, J = 7.2 Hz, 3-H), 7.79 (1H, t, J = 7.2 Hz, 2-H), 8.22 (1H, d, J = 8.0 Hz, 4-H), 8.59 (1H, d, J = 8.0 Hz, 1-H). ^{13}C NMR δ_C (CD_3OD, 100.6 MHz), 11.81 ($NHCH_2CH_2CH_3$), 14.64 ($N(CH_2CH_2CH_2OCH_2CH_3)_2$), 23.13 ($NHCH_2CH_2CH_3$), 23.38 ($N(CH_2CH_2CH_2CO_2Et)_2$), 31.51 ($N(CH_2CH_2CH_2CO_2Et)_2$), 47.53 ($N(CH_2CH_2CH_2CO_2Et)_2$), 52.0 ($NHCH_2CH_2CH_3$), 61.82 ($N(CH_2CH_2CH_2CO_2CH_2CH_3)_2$), 94.59 (C-6), 97.42 (C-8), 116.55 (C-10), 123.95 (2 × Ar–C), 124.53 (C-4), 125.47 (C-1), 131.06 (Ar–C), 132.10 (Ar–C), 133.04 (C-2), 133.80 (C-11), 135.15 (Ar–C), 149.11 (Ar–C), 152.86 (Ar–C), 155.80 (C-5), 159.37 (C-9), 174.73 (CO_2Et) ppm. HRMS: m/z (EI): Found $[M+1]^+$: 532.28056; $C_{31}H_{38}N_3O_5$ requires $[M+1]^+$: 532.28060.

2.5. Procedure for the synthesis of CdTe quantum dots

A 6×10^{-4} M solution of cadmium nitrate was deoxygenated by bubbling nitrogen for 30 min. Then, a 0.02 M aqueous solution of mercapto-propionic acid (MPA) was added in order to achieve a [MPA]/[Cd] molar ratio of 1.1. An appropriate amount of a polytelluride solution, with a concentration of 0.2 M in telluride, was added and the resulting solution was heated at 85 °C during 1 h. The polytelluride solution was obtained from tellurium powder upon addition of hydrazine and a 25% solution of sodium methoxide in methanol. The resulting CdTe QDs aqueous solution had an

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excitonic absorption peak at 575 nm and an emission at 620 nm with a fluorescence quantum yield of 0.065.

2.6. Typical procedure for the coupling of CdTe quantum dots with benzo[a]phenoxazinium chloride **6d**

A solution of CdTe QD's in water (1 mL, ~6×10^{-4} M) was pippeted to an eppendorf tube, and *N*-(3-dimethylaminopropyl)-*N*́-ethylcarbodiimide hydrochloride (EDC) (0.1917 mg) was added. The above solution was heated at 40 °C for 15 min, and then a 0.1 mM ethanolic solution of benzo[*a*]phenoxazinium chloride **6d** (200 μL) was added and the reaction mixture was heated at the same temperature with continuous stirring for 1 h. After the prescribed time, the reaction mixture was cooled to room temperature and centrifuged (10,000 rpm for 15 min). The QDs conjugated with compound **6d** precipitated from the solution. The supernatant liquid was discarded and the quantum dots were washed thoroughly with distilled water for 3 times and the residual particles remained in the bottom were dried and used for further physical studies.

2.7. Typical procedure for drying of ethanol

The reagent grade absolute ethanol was further dried in accordance with a reported procedure [31]. A 3 Å molecular sieves (50 g) was weighed in a round bottomed flask containing a reagent grade absolute ethanol (250 mL). The flask was closed tight and ethanol was left to stand over the sieves for 5 days. The dry ethanol was filtered by a Whatman resist 13/0.45 PTFE filter unit and used for the photophysical measurements.

2.8. Typical procedure for preparation of buffer solutions of variable pH

Buffer solutions of pH = 2, 7, 11 were prepared following the procedure reported in Ref. [32], by mixing appropriate amounts of a mixed acidic solution (0.2 M boric acid and 0.05 M citric acid) and a 0.1 M sodium phosphate solution.

3. Results and discussion

3.1. Synthetic methods

Benzo[*a*]phenoxazinium chlorides **6a**–**f** and **7** were obtained by the condensation reaction of nitrosophenol precursors **3a**,**b** and **4** with naphthyl derivatives **5a**–**d**. The selection of these precursors is important, since they determine the substituents present at the 5- and 9- positions of the tetracyclic aromatic system of the target compounds.

The reaction of 3-aminophenol with 3-bromopropan-1-ol followed by silica gel column chromatography purification afforded the mono- and di-*N*-alkylated derivatives, namely 3-((3-hydroxypropyl)amino)phenol **1a** and 3,3′-((3-hydroxyphenyl)azanediyl)bis(propan-1-ol) **2a**, respectively. Similarly, 3-aminophenol was reacted with ethyl 4-bromobutanoate to afford ethyl 4-((3-hydroxyphenyl)amino)butanoate **1b** and diethyl 4,4′-(3-hydroxyphenyl)azanediyl)dibutanoate **2b** in moderate yields. By the nitrosation of above compounds with sodium nitrite in hydrochloric acidic solution [33], the required 5-((3-hydroxypropyl) amino)-2-nitrosophenol hydrochloride **3a**, ethyl 4-((3-hydroxy-4-nitrosophenyl)amino)butanoate hydrochloride **3b**, and diethyl 4,4′-((3-hydroxy-4-nitrosophenyl)azanediyl)dibutanoate hydrochloride **4** was obtained.

The intermediates **5**, namely *N*-propylnaphthalen-1-amine **5a**, N^1-(naphthalen-1-yl)propane-1,3-diamine hydrobromide **5b**, 3-(naphthalen-1-ylamino)propan-1-ol **5c** and ethyl 4-(naphthalen-1-ylamino)butanoate **5d** possessing the methyl, amine, hydroxyl and ester groups as terminals of the aliphatic chain was achieved by alkylation of naphthlen-1-amine with 1-bromopropane, 3-bromopropan-1-amine hydrobromide, 3-bromopropan-1-ol and ethyl 4-bromobutanoate respectively, in ethanol under reflux conditions as previously reported [25,27,28]. After purification by silica gel column chromatography, intermediates **5a**–**d** were isolated as oils and solid (**5a**,**b**) in moderate yields, and spectroscopic data confirmed the expected structures, being in accordance with data published [25,27,28].

The reaction of 5-((3-hydroxypropyl)amino)-2-nitrosophenol hydrochloride **3a** with *N*-propylnaphthalen-1-amine **5a** or N^1-(naphthalen-1-yl)propane-1,3-diamine hydrobromide **5b** in an acidic medium (concentrated hydrochloric acid), using ethanol as solvent, and after silica gel column chromatography purification gave the corresponding benzo[*a*]phenoxazinium chlorides **6a**,**b**, possessing the hydroxyl groups as the terminal substituents at the 9-positions and methyl or amine in the 5-position, respectively. Similarly, reaction between ethyl 4-((3-hydroxy-4-nitrosophenyl) amino) butanoate hydrochloride **3b** and *N*-propylnaphthalen-1-amine **5a**, N^1-(naphthalen-1-yl)propane-1,3-diamine hydrobromide **5b**, 3-(naphthalen-1-ylamino)propan-1-ol **5c** or ethyl 4-(naphthalen-1-ylamino)butanoate **5d** resulted in compounds **6c**-**f**, with an ester functionality at 9-position and methyl, amine, hydroxyl and ester groups as the terminal substituents at the 5-position of the benzo[*a*]phenoxazine core.

In addition, 9-position bifunctionalised benzo[*a*]phenoxazinium chloride **7** was also obtained by using 3 diethyl 4,4′-((3-hydroxy-4-nitrosophenyl)azanediyl)dibutanoate hydrochloride **4** and *N*-propylnaphthalen-1-amine **5a**. These new set of compounds were obtained as blue solids in moderate yields and were fully characterized by high resolution mass spectrometry, IR and NMR (^{1}H and ^{13}C) spectroscopy (Scheme 1).

The ^{1}H NMR spectra showed the methyl group for the carboxylic ester functionality (**6c**-**f**, **7**) as a triplet (1.29–1.33 ppm) and the aliphatic methyl (**6a**, **6c**, **7**) (δ 1.13 or 1.14 ppm), the methylene protons directly linked to the nitrogen atom at 5- and 9-positions (NH*CH*$_2$ or N*CH*$_2$) displayed as triplets, broad singlets and multiplets centred around (δ 3.26–3.88 ppm), as well as the adjacent methylene groups, NHCH$_2$*CH*$_2$ or NCH$_2$*CH*$_2$ as multiplets and broad singlet (δ 1.37–2.31 ppm), and NHCH$_2$CH$_2$*CH*$_2$ or NCH$_2$CH$_2$*CH*$_2$, which appeared as triplets, broad singlet and multiplet (δ 2.37–3.75 ppm). In addition to the signals corresponding to the aliphatic N-substituents in the heterocycle, the ^{13}C NMR spectra displayed the expected aromatic carbons, that we highlighted C-8 (δ 93.96–97.42 ppm), C-6 (δ 95.13–97.21 ppm), C-11 (δ 131.18–134.11 ppm) and C=O (δ 174.73–175.21 ppm). Bands of the ester C=O bond (1718–1731 cm^{-1}), hydroxyl and amine (3233–3419 cm^{-1}) groups, as well as a strong band of the C=N bond (1545–1549 cm^{-1}) due to the fused oxazine ring were observed in their IR spectra.

3.2. Photophysical studies

From our previous research work [8] it was found that, in ethanolic medium, the benzo[*a*]phenoxazinium chlorides show acid–base equilibria between the benzo[*a*]phenoxazinium cation (AH^+) and the corresponding deprotonated neutral form (A) which manifests itself as spectral variations with dye concentration. The basic form is characterized by an absorption band around 500 nm and a broad emission band near 600 nm with low fluorescence quantum yield (~0.01) while the acidic form showed absorption near 620 nm and emission around 650 nm with high fluorescence quantum yield (>0.1). In aqueous medium, non-emissive H-aggregates of the acidic form are observed as a blue shoulder around

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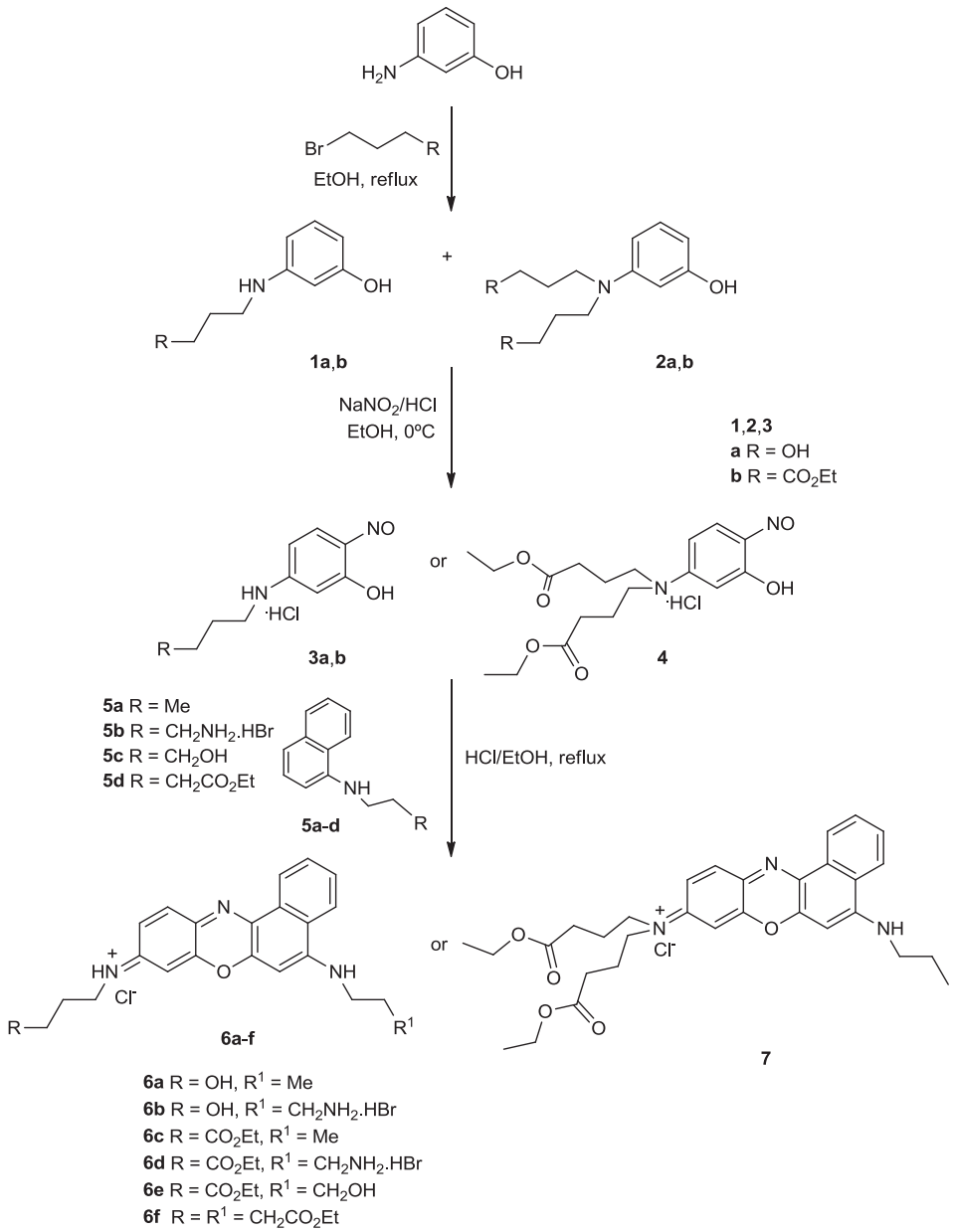

Scheme 1. Synthesis of benzo[*a*]phenoxazinium chlorides **6a**–**f** and **7**.

600 nm. Recently [22], the presence of a tautomeric equilibrium (proton displacement resulting in localization of the positive charge in one of the 5- or 9-amino positions) was shown to occur when these compounds were tested in water-in-oil (w/o) microemulsions. The tautomer loses a part of the π conjugation and appeared near 540 nm in the emission spectrum. For benzo[*a*] phenoxazinium chlorides with non-functionalized terminals [34] it was possible to obtain the deprotonation equilibrium constant in absolute ethanol.

Benzo[*a*]phenoxazinium chlorides (**6a**–**f** and **7**) reported in this work exhibit absorption spectrum in ethanol that varies with dye concentration showing a different proportion of acidic and basic form, but in an inconsistent and irreproducible way (data not shown). Also ethanol from different sources resulted in distinct spectra. Considering, the amount of water dissolved in absolute ethanol depends on ethanol batch and on atmospheric conditions, we decided to dry the ethanol prior to its use. Overall, a different colour was observed in all the solutions. The solutions turned reddish instead of the blue colour typically observed in "reagent grade absolute" ethanol.

Electronic absorption and emission spectra of 4×10^{-6} M solutions in dried ethanol and in buffered aqueous solutions of the fluorophores **6a**–**f** and **7** was then measured. The ethanolic media was either acidified with trifluoroacetic acid (TFA) or basified by methanolic solution of tetraethylammonium hydroxide (TEAH) at 1.5 M. Table 1 shows summarized data of this study alongside with Figs. 1–3. The fluorescence quantum yields were determined in acidic media at 575 nm excitation in order to maximize the contribution of the AH^+ form in both emission and absorption spectra. Oxazine 1 was used as a standard ($\Phi_F = 0.11$ in ethanol) [35] at 575 nm excitation. Table 1 shows that compound **7** has the greatest absorption and emission maxima. This is mainly a result of the double alkylation in the 9-amino position as previously observed [8,28]. Compounds **6b** and **6f** showed the highest

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Table 1
Yields and photophysical data of compounds **6a**–**f** and **7** in ethanol acidified with trifluoroacetic acid (TFA) and buffered aqueous solution at pH = 2 ($C = 4 \times 10^{-6}$ M).

Cpd	Dry ethanol acidified with TFA						Buffer solution pH = 2				
	Yield (%)	λ_{abs} (nm)	ε ($M^{-1}cm^{-1}$)	λ_{em} (nm)	$\Delta\lambda$ (nm)	Φ_F	λ_{abs} (nm)	ε ($M^{-1}cm^{-1}$)	λ_{em} (nm)	$\Delta\lambda$ (nm)	Φ_F
6a	21	615	77,375	647	32	0.39	606	39,250	650	44	0.21
6b	53	629	10,850	646	17	0.42	619	5650	652	33	0.16
6c	59	623	97,925	648	25	0.37	615	45,500	653	38	0.15
6d	68	626	66,100	646	20	0.29	620	48,775	652	32	0.17
6e	47	623	87,900	647	24	0.38	617	46,175	653	36	0.15
6f	44	625	77,025	647	22	0.41	619	54,025	654	35	0.14
7	50	634	125,950	671	37	0.31	631	66,650	677	46	0.55

fluorescence efficiency in ethanolic media, while in water the dialkylated compound **7** is the one with highest quantum yield. Notably, a greater Stokes shift in aqueous media was also observed which clearly indicates the stronger solvent–fluorophore interactions.

Fig. 1(A) shows emission spectra at 575 nm excitation, where only the acidic AH^+ form is excited, whereas Fig. 1(B)–(D) plot emission spectra at 470 nm excitation where other possible molecular forms of the studied benzo[*a*]phenoaxine compounds can also contribute to the fluorescence spectrum. In dry ethanol medium at 470 nm excitation, the acidic form fluorescence is still observed, but depending on the structure of the compound, other bands of different intensities also appear. Near 600 nm for compounds **6a**, **6b**, **6e** and **7** and near 580 nm for compounds **6c**, **6d** and **6f**. Fig. 1(C) and (D) shows emission and excitation spectra either in acidic (Fig. 1(C)) or basic (Fig. 1(D)) conditions. These bands are only seen in basic conditions (Fig. 1(D)) and, from excitation spectra further distinction is observed for compounds **6c**, **6d** and **6f** through a red shift (540 nm instead of the usual ~500 nm) and sharper absorption of the expected neutral form. The only distinctive structural characteristic of these compounds is the existence of ethyl ester terminals in 9-amino position, with compound **6f** having an additional one in the 5-amino position. The exceptions are compound **6e** that either have a very small effect or the presence of the hydroxyl group in the other end (5-amino position) disrupts this specific interaction and compound **7** where there are two ethyl ester terminals in 9-amino position transforming this amine into a tertiary one.

Under the applied acidic conditions (Fig. 1(C)) it is seen that, with an exception of compound **7**, it was not possible to completely displace the acid–base equilibria. Further, an additional emission is observed near 540 nm especially for compound **6f**. According to a previous study this band probably corresponds to a tautomeric form with the excitation spectrum showing absorption at ~510 nm and ~480 nm.

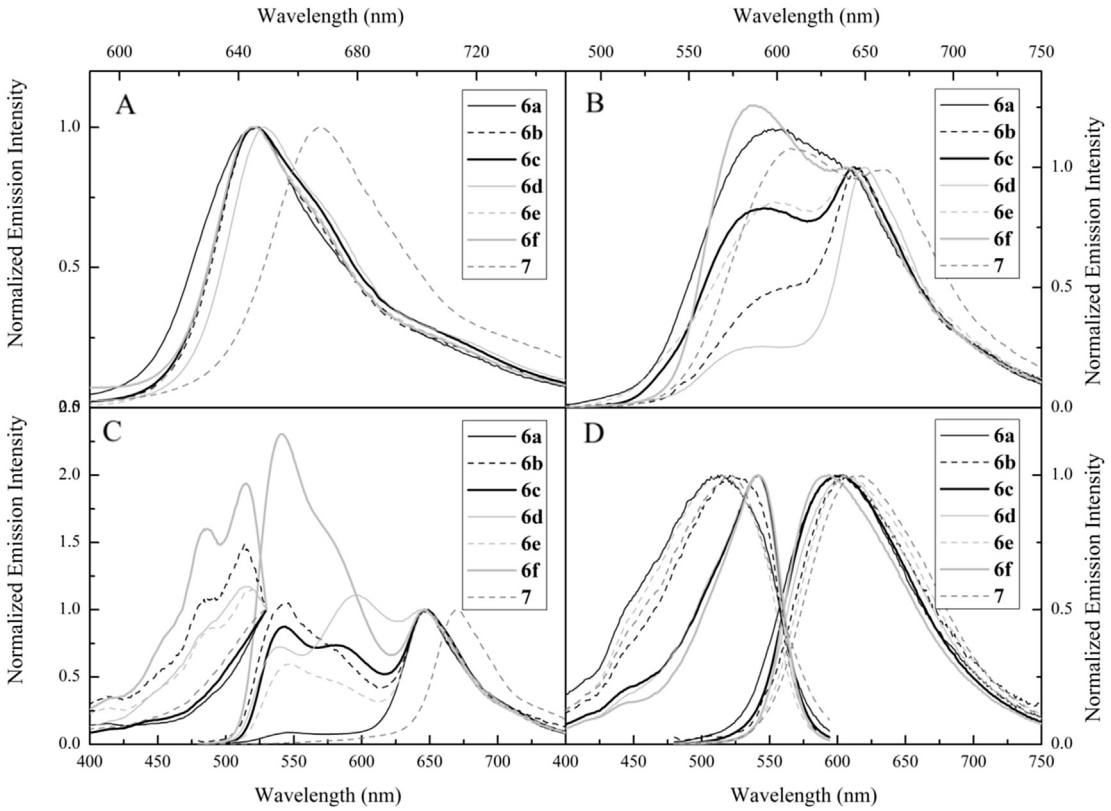

Fig. 1. Emission and excitation spectra of compounds **6a**–**f** and **7** in dried ethanol media. A – In dried ethanol with excitation at 575 nm; B – In dried ethanol with excitation at 470 nm; C – In acidified dried ethanol at 470 nm excitation; D – In basified dried ethanol at 470 nm excitation; the excitation spectra were collected at 605 nm emission wavelength.

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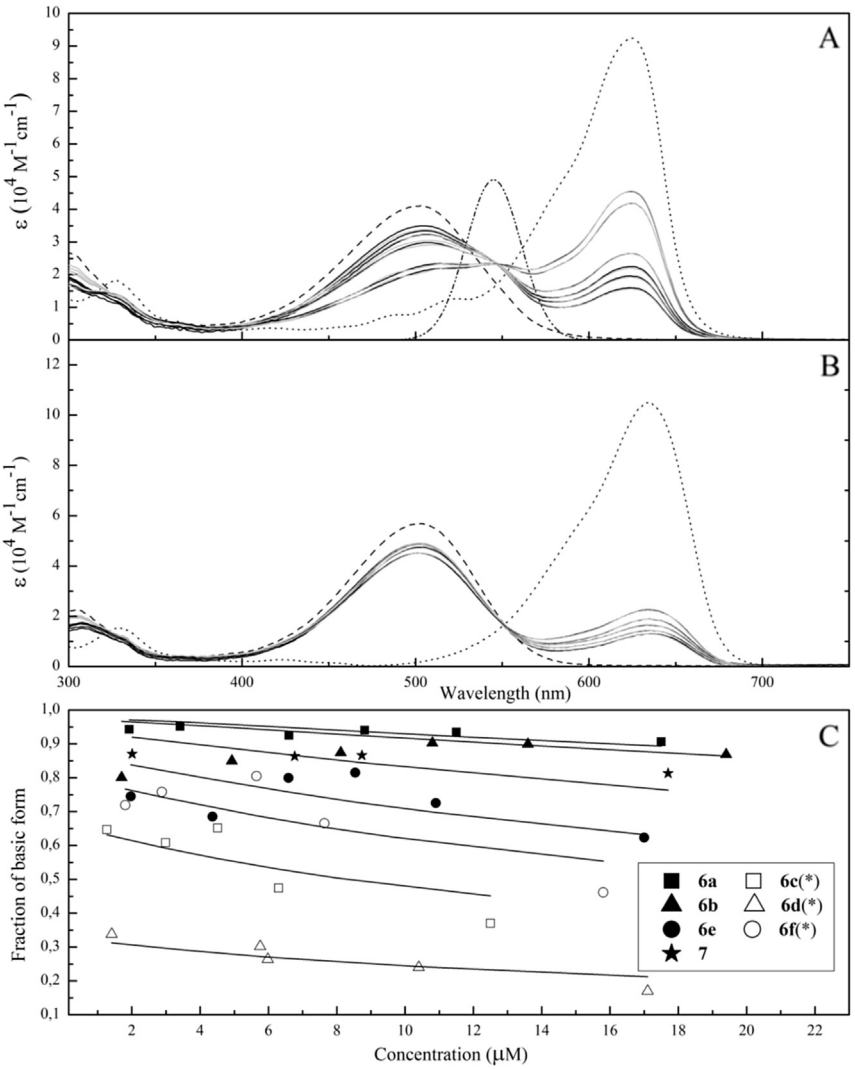

Fig. 2. Absorption spectra of compounds **6f** (A) and **7** (B) in dried ethanol media at concentrations from 2 μM to 20 μM (solid lines – experimental; grey lines – fitted spectrum); the dotted line is the spectrum of 4 μM concentration in acidified ethanol; the dashed line is the spectrum correspondent to the basic form that, in the case of compound **6f** (A) was corrected with a fraction of the spectrum represented by the dash-dotted line. Panel C shows the obtained fraction of basic form of compounds **6a**–**f** and **7** in dried ethanol media. (*) Compounds in which an additional band around 540 nm was required in the fitting procedure.

Fig. 2 shows molar absorptivity of compounds **6f** (panel A) and **7** (panel B) as a function of dye concentration. In the case of compound **7** the spectra could be fitted to a sum of a fraction of the spectrum obtained in basic conditions (dashed line) with the complementary fraction of the spectrum obtained in acid (dotted line) conditions. The same was possible for compounds **6a**, **6b** and **6e** (data not shown). However, for compound **6f** (Fig. 2(A)) an additional sharp absorption near 540 nm band had to be taken into account. The spectrum in basic conditions was a sum of the spectrum plotted as a dashed line with 12% of the band at 540 nm (dash-dotted line). The criteria used to obtain this value was that the resulting basic form spectrum (dashed line) should be similar to that observed for compound **7**, namely with a smooth descent. It was then possible to fit the experimental spectra a sum of three components: the acid form; the corrected basic form and the additional benzo[*a*]phenoxazine molecular form that corresponds to the 540 nm sharp band. Interestingly, the absorption bands corresponding to the tautomeric form (~520 nm and 480 nm) were not apparent which mean that their concentration is very low. The same procedure was successful for compounds **6c** and **6d** (data not shown) with a contribution of, respectively, 12% and 9% of the additional band to the spectrum obtained in basic conditions. Considering the sharpness of the additional absorption band at 540 nm and corresponding emission at 580 nm it is possible to put forward the possibility of J-aggregate formation of the neutral form of compounds **6c**, **6d** and **6f** promoted by the presence of ethyl ester groups, especially in the 9-amino position.

Supposing that the neutral basic molecular population is in equilibrium, as a whole, with the acid cationic subpopulation, it is possible to estimate a dissociation equilibrium constant from the variation basic form fraction with compound concentration. Following a procedure similar to that used in a previous study [34] the fitted curves in Fig. 2(C) are defined by the equilibrium dissociation constants, K_a, shown in Table 2 and by an ethanol self-dissociation constant of 1.75×10^{-11} M^2, obtained globally for all data in Fig. 2(C). This value is higher than expected as for methanol the corresponding value is 1.2×10^{-17} M^2 [36]. This could be the consequence of specific interactions of the studied benzo[*a*]phenoxazine dyes with ethanol solvating molecules.

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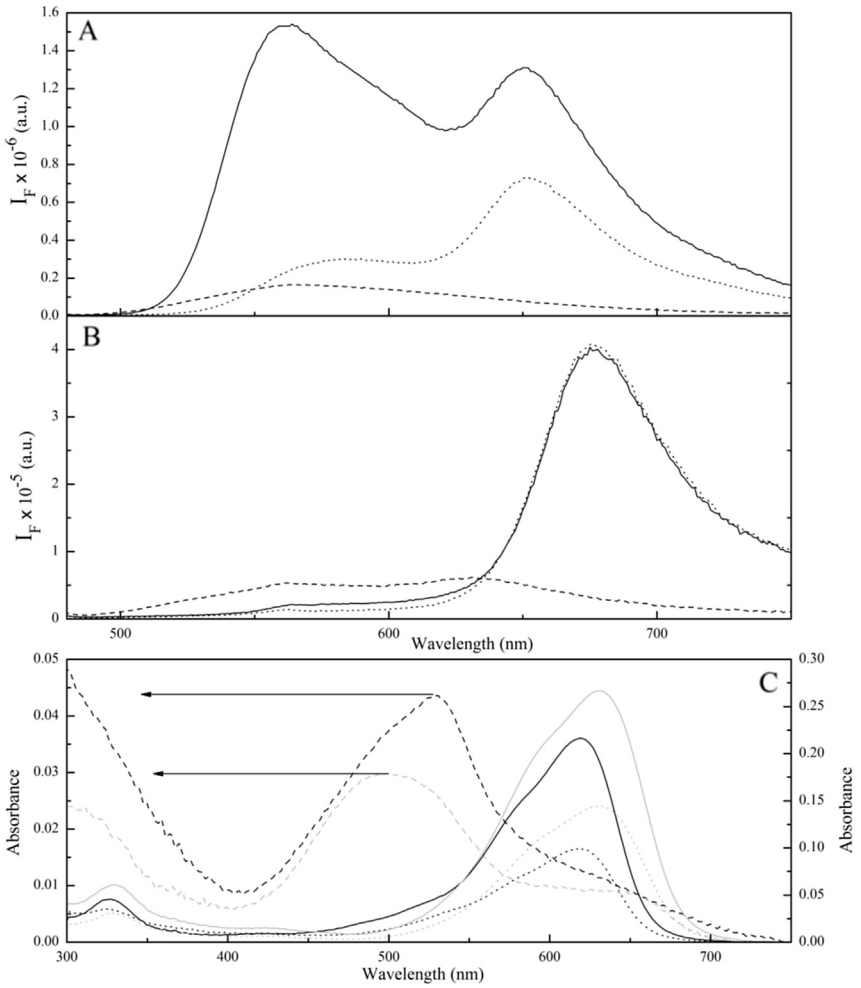

Fig. 3. Emission spectra at 470 nm excitation of compounds **6f** (A) and **7** (B) in aqueous buffered solutions at pH = 2 (solid line) pH = 7 (dotted line) and pH = 11 (dashed line). Panel C shows the absorption spectra of compound **6f** (black lines) and compound **7** (grey lines) at pH = 2 (solid line), pH = 7 (dotted line) and pH = 11 (dashed line).

The obtained values of K_a show that the presence of an OH terminal in 9-amino position (compounds **6a** and **6b**) favours the dissociation process. This is consistent with a main deprotonation site at 5-amino position as the OH group would stabilize the localization of positive charge at the 9-amino position. In previous work [37] additional evidence for preferential deprotonation at the 5-amino position was already given. The presence of NH_2 terminal at 5-amino position disfavours deprotonation as can be concluded by comparing compounds **6b** and **6a**. On the contrary, by comparing compound **6c** with **6e** and **6f**, the presence of an OH or an ethyl-ester group at the 5-amino position seems to increase K_a. Compound **7** can only be deprotonated at 5-amino position and the disubstitution with ethyl ester terminals seems to increase the dissociation constant (K_a(**7**)>K_a(**6c**)).

Fig. 3 shows the emission at 470 nm excitation and absorption spectra of compounds **6f** and **7** in aqueous media at controlled pH values using suitable buffers. Huge changes with pH are observed. At pH = 2, the tautomeric form emission appears for compound **6f** but in much less amount for compound **7**. This is consistent with the result in acidified ethanol media (Fig. 1(C)). In basic conditions (pH = 11) low emission is observed. While for compound **6f**, the band at ~580 nm is still observed but 10 times less intense than in basified ethanol (Fig. 1(D)), for compound **7** there is practically no emission in the 500–620 nm spectral region. The absorption spectra plotted in Fig. 3(C) show that there is much less absorption in basic than in acidic conditions. Further the fact that the baseline is inclined indicates the presence of light dispersion that originates from extended aggregation of neutral benzo[*a*]phenoxazine molecules that are expected to have low solubility in water. The additional band at 540 nm is now much more pronounced for compounds **6f** than what was observed in basified ethanol (Fig. 2(A)). This constitutes further evidence that this band can correspond to J-aggregates of neutral benzo[*a*]phenoxazine molecules.

As the studied compounds show high environment sensibility and have functional groups that can be used to couple with other molecular entities and following recent published research on the behaviour of Nile Blue when conjugated with quantum dots [20], initial attempts on coupling bifunctional benzo[*a*]phenoxazine derivatives with CdTe quantum dots was performed.

The CdTe quantum dots were prepared in aqueous media using mercapto-propionic acid (MPA) as a capping agent. It was expected

Table 2
Equilibrium dissociation constants of compounds **6a**–**f** and **7** in dried ethanol.

Compound	**6a**	**6b**	**6c**[a]	**6d**[a]	**6e**	**6f**[a]	**7**
K_a ($10^{-5}M^{-1}$)	14.2	11.5	1.28	0.32	2.19	2.08	4.95

[a] Obtained considering an additional absorption band near 540 nm.

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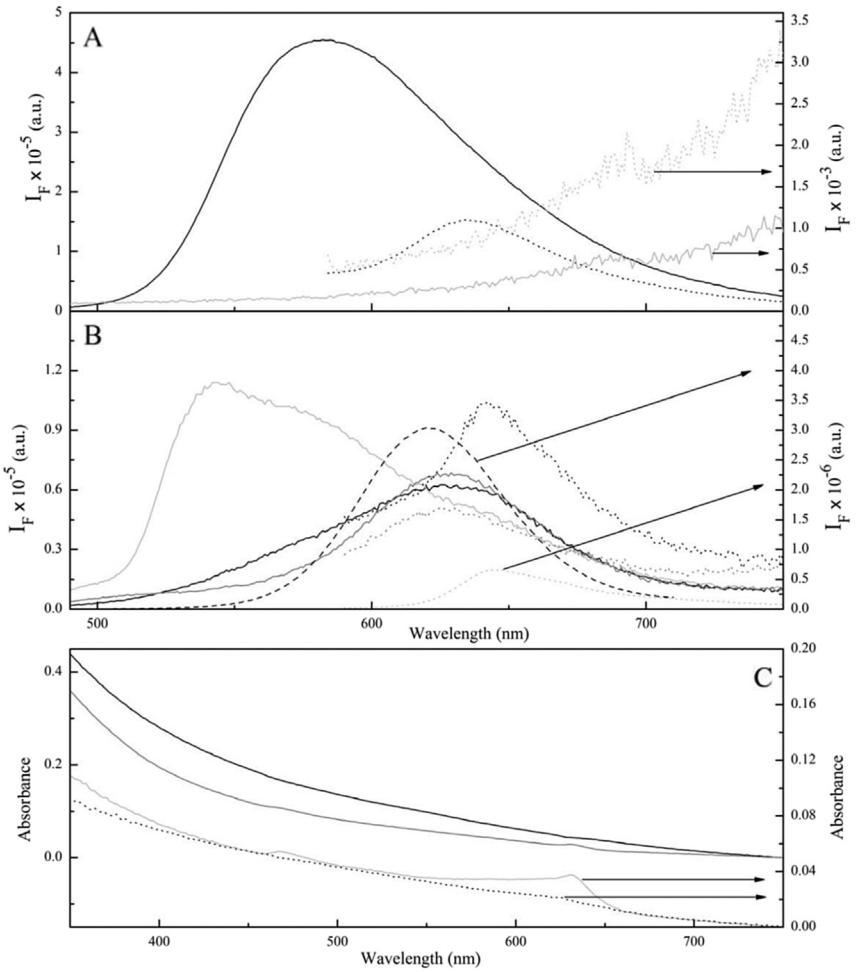

Fig. 4. A – Emission spectra at 470 nm excitation (solid lines) and at 575 nm (dotted lines) of the 1st (black lines) and 2nd (grey lines) supernatant resulting from washing CdTe-**6d** quantum dot conjugates. B – Emission spectra at 470 nm excitation (solid lines) and at 575 nm (dotted lines) of CdTe-**6d** quantum dot conjugates in dried ethanol (black lines), acidified ethanol (grey lines) and basified ethanol (dark grey lines). The dashed black line corresponds to CdTe quantum dots emission. C – Absorption spectrum of CdTe-**6d** quantum dot conjugates in dried ethanol (black line); acidified ethanol (grey line); basified ethanol (dark grey line) and in water at pH = 4 (dotted line).

that the carboxylic acid functional groups at the surface of the quantum dot can be activated and coupled with aliphatic terminal amines using standard peptide coupling procedures. In this context, compound **6d** was chosen and additionally to the amine terminal at the 5-amino position, the chemical function at the 9-amino position is relatively inert in the coupling reaction conditions. Fig. 4(A) shows the emission of the aqueous media obtained upon successive particle centrifugation, supernatant removal and water addition. It can be concluded that any benzo[*a*]phenoxazine emission arising from the resulting particles results from attached molecules rather than from physically adsorbed ones. Fig. 4(B) shows that the CdTe emission is extensively quenched by the proximity of **6d** molecules probably through a photoinduced electron transfer process. In acid conditions the remaining emission of CdTe quantum dots is completely lost, whereas it is still observable in neutral and basic conditions. This is an indication that energy transfer from the excited quantum dots to the acid form of the benzo[*a*]phenoxazine occurs. The results also showed the response of CdTe-**6d** conjugate was highly dependent on pH of the medium.

The absorption spectra in Fig. 4(C) showed less light scattering in acidified ethanol medium and in water at pH = 4. This indicates that when compound **6d** molecules are protonated there is a lower tendency to quantum dot aggregation although the reverse effect is expected from the protonation of the remaining carboxylic acid groups at the CdTe surface. Also very noticeable is the absorption band of compound **6d** when the CdTe-**6d** conjugates are in acidified ethanol, although in water at pH = 4 it almost disappear.

4. Conclusion

A new set of (bi)functionalized benzo[*a*]phenoxazinium chlorides possessing carboxylic ester, hydroxyl and amino functional groups was synthesized. The photophysical behaviour of these compounds was evaluated in dry ethanol and it was found to be distinct from solvent grade absolute ethanol. A benzo[*a*]phenoxazinum chloride possessing an amine functional group at the terminal of the 5-amino position was coupled with CdTe quantum dots and its photophysical studies revealed the evidence of photoinduced electron and energy transfer to the attached dye. This type of conjugated systems can function as pH sensor and/or for measuring the NADH levels.

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