## Photophysical properties and immobilisation of fluorescent pH responsive aminated benzimidazo[1,2-*a*]quinoline-6-carbonitriles

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## Graphical abstract



## Highlights

* Photophysical data of amino-substituted benzimidazo[1,2-*a*]quinoline-6-carbonitriles are presented.
* Systems **1**–**4** are unionized at neutral pH and get monocationic at lower pH values.
* The introduced amino groups increase basicity especially at position 5.
* Immobilisation of selected systems further increases the basicity by 2–4 p*K*a units.
* pH sensing and H+ fluoroionophoric potential in bulk optode membranes were revealed.

## Abstract

This work presents a systematic evaluation of 2-amino, 5-amino and 2,5-diamino substituted benzimidazo[1,2‑*a*]quinoline-6-carbonitriles as novel pH probes with a potential application in pH sensing materials or as H+ fluoroionophores in bulk optode membranes. The study was carried out by varying the length, type and position of amino substituents in ten fluorescent dyes with the same benzimidazo[1,2-*a*]quinoline-6-carbonitrile core. The photophysical and acid-base properties of the dyes were investigated by UV/Vis absorption and fluorescence spectroscopies, and interpreted by electronic structure DFT calculations. pH sensing mechanisms and structure-property relations affecting the fluorescence response were discussed through a detailed analysis of the piperidine substituted derivatives **1**–**4**. *Push*–*pull* donor–acceptor interactions stimulate strong fluorescence in the visible spectral range (up to *Φ* = 0.65 for **7**) and induce significant changes in the photophysical properties associated with the acid-base equilibria (up to a 50-fold increase in the fluorescence intensity). p*K*a values determined in aqueous and mixed solutions (*v*/*v* H2O:EtOH 99:1, 50:50), appear suitable for monitoring acidic pH in solution. The most promising candidates were immobilised in thin polymer matrices by the spin coating technique to form fluorescent sensing materials – optodes, and examined as novel pH-sensitive fluoroionophores. In such liquid membrane environment, dyes exhibited significant shift of the apparent p*K*as by almost 4 units towards higher values. Bright and blue emissive thin films showed pH response and dynamic range around p*K*a = 5, making it suitable for a wide range of optical sensing applications.

**Keywords:** benzimidazole; quinoline; pH probe; pH sensor; fluorescence; fluoroionophore

## 1. Introduction

Design and development of small molecules for (chemo)sensing and optoelectronic applications is of great interest in organic chemistry and sensing technology. Heterocyclic molecular systems with pronounced and diverse spectral responses are among the most extensively studied classes of organic compounds. Structural similarity with naturally occurring compounds enabled the applications of such molecules in the selective recognition of biomolecules, in molecular biology or medicinal chemistry [1]. For example, benzimidazoles are biologically important purine-like heterocyclic compounds widely studied for their therapeutical activity and exploited as pharmacophores [2]. Benzimidazole derivatives show a broad range of biological activities including antibacterial, antitumor, antifungal or antiviral properties [3-4]. However, the multifunctionality of the benzimidazole core enables many other applications of its derivatives in different fields, such as optoelectronics [5, 6], fluorescent probes for detection of biologically relevant molecules [7], efficient and selective sensors for cations or anions [8-10], as well as for metallochemistry ligands. A number of experimental and theoretical studies of benzimidazole-based compounds have been published in order to examine and exploit their functional properties, mainly focusing on the protonation equilibria in aqueous solutions, metal-ion complexation or solvatochromism [11-13].

Benzannulated benzimidazoles, such as benzimidazo[1,2-*a*]quinolines, possess a *π*-conjugated planar structure exhibiting bright fluorescence and enabling their application as fluorescent chemosensing molecules, organic luminophores or fluorescent dyes [14, 15]. Their substituted derivatives are ideal candidates for the formation of *push-pull* or intramolecular charge transfer (ICT) states, providing properties useful in optical sensing applications, such as the shift of the emission wavelength and the quenching or enhancement of the fluorescence intensity in the presence of potential analytes [16-18].

Fluorescent sensors have been actively investigated in recent years because of their high sensitivity and rapid responses to various analytes [19, 20]. Fluorescent molecular pH probes have been developed for a wide range of pH scale, from extremely acidic to extremely basic conditions. More importantly, functional materials based on novel fluorophores are attractive and widely utilised for high-tech innovations and other modern applications. Development of fluorescent pH sensing materials employing novel sensitive dyes is a constantly growing scientific field in which benzimidazole and quinoline derivatives play an important role [21-23].

Fluorescent derivatives of benzimidazo[1,2-*a*]quinolines reveal an enormous potential for biological applications, such as fluorescent imaging or intracellular pH sensing [24-27], which is another reason for their further study and implementation. Typical structure of sensor molecules based on ICT has adequately positioned electron donating (–NR2 or –OR) and electron withdrawing (–NO2 or –CN) groups linked by a *π*-conjugated system (D-*π*-A or *push-pull* systems). Additionally, fluorescence properties of such systems can be significantly modified by multiple substituents placed at different positions of the planar chromophore. As an illustrative example, our previous work on 2-amino-5-phenylbenzimidazo[1,2-*a*]quinoline-6-carbonitrile revealed its exceptional selectivity towards Zn2+ ions [28], suggesting that such 2-amino-6-cyano substituted frameworks involve *push-pull* aromatic features, which makes them attractive candidates for sensing applications. This motivated us to further investigate possible applications of these multifunctional compounds. Accordingly, here we carried out a systematic study of ten different 2-amino, 5-amino and 2,5-diamino substituted benzimidazo[1,2-*a*]quinoline-6-carbonitriles focusing on their structure-property relations and photophysical characterisation, while evaluating the influence of pH on their absorption and fluorescence properties in order to develop novel pH probes. The best candidates were immobilised in thin polymer matrices by the spin coating technique, and we demonstrated their H+ fluoroionophoric potential for the development of bulk optode membranes and novel pH sensing materials.

## 2. Materials and methods

**2.1. Reagents and instrumentation**

All chemicals and solvents for the synthesis were purchased from commercial suppliers Acros, Aldrich or Fluka. Methanol, hydrochloric acid, sodium hydroxide and organic solvents were obtained from Kemika d.d., Zagreb. Milli-Q water was used for the preparation of aqueous solutions. pH in the range 1–13 was measured on a commercially available combination pH electrode BlueLine 17 pH (Schott AG, Mainz, Germany). Absorbance spectra were recorded on the Carry 100 Scan Varian spectrophotometer. Fluorescence measurements were carried out on the Varian Cary Eclipse fluorescence spectrophotometer. Thin films were prepared by using high-molecular-weight poly(vinyl chloride) (PVC), dioctylsebacate (DOS), tetrahydrofuran (THF) and potassium tetrakis(4-chlorophenyl)borate (PTCB), all supplied by Sigma–Aldrich, the ultrasonic bath (Transsonic T460/H, Elma Hans Schmidbauer GmbH&Co. KG, Singen, Germany) and the spin-coater (KW-4 A spin coater, Chemat Technology Inc., Northridge, CA, USA).

**2.2. Synthesis of aminated benzimidazo[1,2-*a*]quinoline-6-carbonitriles**

All compounds presented in Figure 1 were previously synthesized in our research group by conventional methods for the preparation of similar benzimidazole derivatives [27]. Targeted derivatives substituted with amino side chains of different type and length were prepared from corresponding halogeno substituted benzimidazo[1,2-*a*]quinoline-6-carbonitriles by uncatalyzed microwave assisted amination. Compounds were routinely checked by TLC with Merck silica gel 60F-254 glass plates.

**2.3. Photophysical characterisation and acid-base properties**

Stock solutions were prepared by dissolving the compounds in methanol, *c*(stock)=1×10–3 mol dm–3, from which the working solutions were prepared by dilution. Spectrophotometric titrations were carried out using 1x10–5 mol dm–3 solutions of **1**–**10** in ethanol and/or buffer solutions for the absorbance measurements, and 2x10–6 or 1x10–7 mol dm–3 solutions for the fluorescence measurements. Absorbance measurements were carried out using quartz cells of 1 cm path length. Wavelength scans were performed from 200 to 700 nm, with 1 nm recording resolution. Baseline was recorded prior to each set of experiments. Fluorescence measurements were carried out at 25ºC using 1 cm path quartz cells. Excitation wavelengths were determined from the absorbance maxima. Emission spectra were recorded from 300 to 800 nm. Relative fluorescence quantum yields *Φx* were determined according to eq. (1):

*Φ*x = *Φ*s × *A*s *D*x *n*x2 / *A*x *D*s *n*s2 (1)

wherein *A* is the absorbance at the excitation wavelength, *D* is the area under the corrected emission curve and *n* is the refractive index of used solvents. The subscripts *s* and *x* refer to the standard and the unknown, respectively. The employed standard was quinine sulphate with the reported fluorescence quantum yield of 0.54 [29]. All samples were purged with nitrogen to displace oxygen. The reproducibility (difference between the largest and the smallest value in a series of three independent measurements, divided by their arithmetic mean) of quantum yield measurements was better than 10 %.

**2.4. Immobilisation of compounds**

Plasticised polymer films were prepared according to recommended procedures for ion selective optode membrane preparation [30], with minor modifications. Chemical “cocktail” mixtures were made by dissolving 67 mg of PVC, 140 mg of DOS, 2.2 mg of PTCB and the equivalent amount of dye (molar ratio PTCB:dye 1:1) in 1.5 mL of THF. The mixtures were homogenised for 15 min in an ultrasonic bath and left over night. Thin films were made by the spin-coating technique. Volume of 0.1 mL was deposited onto 2.5 cm x 2.5 cm polyester sheets and the spinning was setup at 1200 rpm for 40 s. Films were stored in dark for at least 3 hours and then cut into 2.5 cm x 0.8 cm strips for the spectrophotometric measurements. pH response was recorder in buffer solutions, with pH = 2–12. Hydrochloric acid and sodium hydroxide were used in the acidic and basic regions as the titration end points (pHs 1 and 13, respectively). Reversibility of demonstrated pH sensor was tested by alternately immersing the same thin film in buffer solutions at pH=3 and pH=8. The absorption and emission spectra were recorded after 4 min. Response time is calculated as an amount of time that it takes for the detector to measure 95% of the maximum analyte level.

**2.5. Computational methods**

Molecular geometries were optimized in the gas-phase using the B3LYP DFT functional employing the 6–31+G(d) basis set in accordance with our earlier work [13, 31]. Free energies were obtained by using thermal corrections from the corresponding un-scaled frequency calculations. Gas-phase results were refined with a single-point energy calculation employing a more flexible 6–311++G(2df,2pd) basis set. To account for the solvation effects, the obtained geometries were reoptimized and the solvation free energies were attained by using the implicit SMD polarizable continuum model [32] with either pure water or ethanol solvent parameters. This computational setup was prompted by its success in reproducing the kinetic and thermodynamic parameters of various organic reactions [33, 34], and predicting accurate p*K*a values for similar organic systems [35]. Aqueous p*K*a values were calculated in an absolute fashion employing the gas-phase proton free energy of *G*º(H+) = 6.28 kcal mol–1 and the experimentally determined value for the proton solvation free energy of Δ*G*SOLV(H+) = –265.9 kcal mol–1 in water [36], consistent with the value used by Truhlar and co-workers in parameterizing the SMD model [32]. Δ*G*SOLV(H+) value includes the free energy change of –1.89 kcal mol–1 associated with moving from a gas-phase pressure of 1 atm to a liquid-phase concentration of 1 M.

Excited state calculations were performed with the TD–DFT approach by using the 6–311++G(2d,2p) basis set and M06 functional, also with implicit SMD solvation, in line with our previous results on similar compounds [13,31]. The electrostatic potential, electronic density and molecular orbital plots were produced by the GaussView 5.0 software [37]. Λ parameter for the excited states [38] was calculated with the program Multiwfn 3.6 [39], while all the other calculations were performed using the Gaussian 16 software [40].

## 3. Results and discussion

### 3.1. Design and general photophysical properties of fluorescent dyes 1-10

Fluorescent dyes **1**–**10** are conjugated molecular systems with rigid benzimidazo[1,2-*a*]quinoline core, as presented in **Figure 1**. Alteration of the fluorescent properties was achieved by modifications on the cyano derived benzimidazo[1,2-*a*]quinoline core. Unsubstituted benzimidazo[1,2-*a*]quinolines-6-carbonitrile **1** is a chemically stable compound with moderate fluorescent efficiency (*Φ* = 0.24). By varying the position of the amino-substituents in **2**-**10**, intramolecular interactions of the examined compounds are significantly affected by different position, type or length of amino-substituents. For example, electron-donating amino-groups extend the conjugation by directly contributing to the conjugated *π*-system with their nitrogen lone pair electrons. Intramolecular interactions caused by the electron withdrawing –CN substituent on the position 6 of the quinoline part are consequently responsible for the formation of *push-pull* molecular system [44]. Such *push-pull* dyes **2**, **5**, **6**, **7** and **8** were further modulated by phenyl or piperidine substituents, placed on the position 5 (dyes **3**, **4**, **9** and **10**). This kind of modification significantly disrupts the charge transfer within the aromatic core and notably decreases fluorescence intensity, as rationalized in Section 3.2 by computations.



**Fig 1.** Derivatives of benzimidazo[1,2-*a*]quinoline-6-carbonitrile.

A systematic comparative study of the spectral properties was preformed, and the general photophysical properties of **1**–**10**, such as the absorption (*λ*abs) and fluorescence maxima (*λ*emiss), extinction coefficients (*ε*) and Stokes shifts (*υ*A – *υ*F), are presented in **Table 1**. We note in passing that some of our current measurements complement those reported earlier [25,28], and, due to different instruments and experimental setups employed to perform the measurements, some discrepancies in molar extinction coefficients and quantum yields can be observed, but we made sure that current results are obtained in a consistent and accurate way.

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**Table 1.** Electronic absorption and fluorescence emission data of the studied compounds **1**-**10** in ethanol*.*

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** |
| **R1** | –H |  | –H |  |  |  |  |  | –H | –NH2 |
| **R2** | –H | –H |  |  | –H | –H | –H | –H |  |  |
| ****abs (nm)** | 348 | 433 | 369 | 397  420 | 409  428 | 410  433 | 409  429 | 409  428 | 353 | 360 |
| ***ε* x 103 (dm3mol-1cm-1)** | 7.89 | 40.82 | 12.22 | 20.88  19.02 | 30.80  34.60 | 26.22  31.91 | 31.38  35.89 | 23.90  23.67 | 12.40 | 12.00 |
| ****emiss (nm)** | 469 | 462 | 486 | 463 | 451 | 458 | 458 | 455 | 480 | 503 |
| **Stokes shift**  **(cm-1)** | 7414 | 1450 | 6524 | 2211 | 1192 | 1261 | 1476 | 1386 | 7495 | 7897 |
| ***Φ*F** | 0.244 | 0.543 | 0.007 | 0.049 | 0.391 | 0.554 | 0.650 | 0.103 | 0.223 | 0.025 |

Since the photophysical features mainly depend on the polarization of the chromophoric system, the parent compound benzimidazo[1,2-*a*]quinoline-6-carbonitrile forms colourless and blue emitting solution, and its amino substituted derivatives are generally yellow and blue-green emitting in solution. The absorption spectra in ethanol show intense donor-acceptor absorption bands in the UV/Vis region. The most intense absorption bands of **1**–**10** lie within 250 – 300 nm (*ε* > 3×104 dm3mol–1cm–1). They are assigned to *π*–*π*\* transitions as confirmed by the computations. In addition, well distinguished bands with *λ*max in the range 350-440 nm (*ε* = 1-4∙104 dm3mol–1cm–1) are observed, and attributed to the *push-pull* nature of the electron donating amino and electron withdrawing cyano substituents. The latter groups extend the conjugated *π*-system, leading to bathochromic and hyperchromic shifts in the absorbance maxima of **2**-**8**, up to 80 nm in respect to the unsubstituted **1**. The shift of a CT-band generally depends on the electronic nature of the substituent. For investigated examples of amino derivatives though, alteration of amino substituents causes no significant wavelength shift of the CT-band. With altering the length of the aliphatic secondary (e.g. dyes **5** and **7**) or tertiary amine (e.g. dye **6**), or introducing cyclic tertiary amine (e.g. dyes **2** and **8**), the wavelengths of the CT absorption maxima remain within 5 nm (from 428 nm to 433 nm) (**Table 1** and **Figures S1-S2**).

The examined dyes exhibit characteristic blue-green fluorescence with a single emission band in the visible region and well-pronounced maxima at *λ* = 480–500 nm (**Table 1**). Excitation spectra of all studied compounds are in good agreement with their absorption spectra. The Stokes shift values suggest moderate differences in the energy upon excitation for 2-amino substituted dyes (1261 – 2211 cm-1), being suitable for diverse applications in optical chemistry and biology. However, the parent dyes without amino substituents in position 2 (**1** and **9**) are colourless and highly fluorescent, showing remarkable Stokes shift (> 7400 cm-1). Such interesting photophysical properties can be exploited in numerous fields, such as sun protection layers or fluorescent brighteners [41]. Quantum yields of the examined 2-amino compounds (**2**, **5**-**8**) are within the range *Φ* = 0.10 – 0.65, indicating bright fluorescence properties and enabling the application in fluorescence microscopy and fluorescent imaging in biology and biochemistry [42,43]. Observed differences in quantum yields may be attributed to many factors. For example, formation of twisted intramolecular charge transfer (TICT) states, especially for dyes with bulky substituents, can strongly affect the relaxation pathways, causing weaker fluorescence.

**3.2. Study of the structure-property correlation**

A study on correlation of structure and spectral properties of benzimidazo[1,2-*a*]quinoline-6-carbonitrile class of compounds was carried out. Compounds **1**-**4** are chosen to be representative examples for this structure-property study due to significant changes in emission properties caused only by the piperidine position (**Figure 2**).



**Figure 2.** A)Effect of piperidine position on the emission properties, *c* = 5×10–7 M of the derivatives **1**–**4**; B) Piperidine substituted derivatives **1**-**4**.

Detailed photophysical characterization of **1**-**4** was conducted by absorbance and fluorescence spectroscopy in solvents of different polarity parameter (**Table S1** and **Figures 3**, **S3–S6**)and by computational methods.

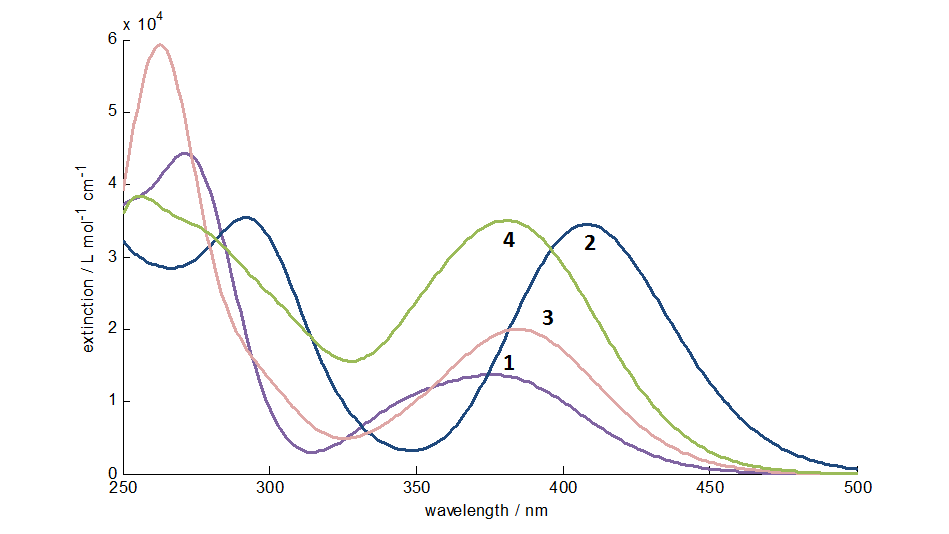
Systems **1**–**4** exhibit bathochromic shifts in absorbance and emission spectra with increasing solvent polarity. Molecular polarity was estimated from Lippert-Mataga plot, constructed by plotting the Stokes shifts of **1**–**4** against the orientational polarizibility of solvents. The plots represent good linear relationship indicating moderate ICT character of molecules, as can be seen for example **2** in **Figure 3b**.



**Figure 3.** A)Normalised emission spectra of **2** in solvents of different polarity; B) Lippert-Mataga plot (Stokes shifts *vs*. orientational polarizibility of solvents) of **2**, *c* = 1×10–7 M, *λ*exc = 433 nm.

Perhaps the most prominent finding is the great difference between the fluorescence intensity in organic solvents and water (**Table S1**). Fluorescence emission in water is up to ten times lower in comparison to ethanol. Reasons of such behaviour, as well as large difference in quantum yields between **1**, **2**, **3** and **4** are further investigated by computational methods (see later). The calculations were utilised to understand the substituent effects on the electronic transitions responsible for the observed UV/Vis spectra.

The calculated UV/Vis absorption spectra are shown in **Figure 4**. They very well reproduce the relative positions and intensities of the absorption bands in the experimental spectra (**Figure S1**). The extent of the charge transfer in the calculated excited states was probed by the parameter Λ, proposed by Tozer et al. as the diagnostic criterion for charge transfer states [38]. **Figure S6** displays Λ plotted against the oscillator strengths *f* of all calculated electronic transitions, to show that for all intense transitions (with *f* > 0.1) the Λ values are above the critical threshold 0.3, which gives reliability to the calculated spectra.



**Figure 4.** The calculated UV/Vis spectra of **1**–**4** in water, obtained with TD–DFT approach utilizing (SMD)/M06/6–311++G(2d,2p) methodology.

The lowest four excited states are examined in more detail as they predominantly determine the absorption spectrum in the visible region. A direct insight into their nature is provided by differences between the excited and ground state electronic densities depicted in **Figure S7**. All these states have *π*–*π*\* character as indicated by the nodal molecular plane and the isodensity surfaces similar to those of the *π*-molecular orbitals (**Figure S8**). The lowest excited states and the frontier MO show a high degree of similarity in all four systems, arising from the common parent benzimidazo[1,2-*a*]quinoline-6-carbonitrile skeleton. Their photophysical properties are mainly determined by this common substructure, modulated by the piperidine substituents attached at different positions.

Electron donor and acceptor substituents coupled to the *π*-conjugated system are known to induce remarkable spectroscopic properties, in particular strong absorption and fluorescence at low excitation energies [44]. Here, the parent skeleton is relatively complex, with four conjugated rings including the imidazole ring and extending to the nitrile group. The effect of introducing the electron donating amino group(s) to the periphery of the complex conjugated system is not quite predictable and straightforward. Coupling with the electron accepting nitrile group should induce electron density shift from the amino group towards the nitrile group, but this also depends on the position of the amino group. The UV/Vis absorption spectra have indicated that the electron density polarization should be strong in **2** and to a lesser extent in **4** and **3**. The ground states of **1**–**4** have large dipole moments in the same order (16.8 D for **2**, 16.2 D for **4**, 12.2 D for **3**, and 11.5 D for **1**), whose directions indicate electron density shift towards the nitrile group. Various schemes were proposed for the quantification of the intramolecular charge transfer, but here it is not clear how to define the relevant molecular fragments in a unique way because the conjugated part between the acceptor nitrile groups and the donating amino groups involves potentially donating imino group. The most comprehensive insight was obtained by plotting the electrostatic potential mapped onto the density isosurface (isovalue 0.0004), shown in **Figure S9**. From these plots it is clear that the electron density polarization is most intense in the excited states of **2**, and followed by **4** and **3**, in full agreement with their absorption spectra. The *push-pull* interactions between the amino and nitrile groups are evident in **2** and **4**, and less discernible in **3**. The reduced donor-acceptor interaction in **3** could be related to the less favourable orientation of the piperidine ring at position 5 in respect to the nitrile acceptor, and to steric repulsion with the nearby atoms (H-atom at position 4 and the nitrile group). The steric repulsion causes partial rotation of the piperidine substituent out of the plane of the main molecular skeleton, which decreases conjugation of the nitrogen atom at position 5 with the central *π*-system, and its coupling with the nitrile group.

The orientation of piperidine at position 5 also plays a role in the quenching of the fluorescence of **3** and likely in **4**. In the first excited state, by geometry relaxation, the piperidine ring in **3** becomes perpendicular to the central *π*-system. This geometry change induces crossing of the lowest excited states, and the first excited state gets very close to the ground state (0.48 eV with solvent relaxation of the excited state). This indicates likely crossing between the first excited and the ground state and the consequential absence of emission due to the nonradiative relaxation. Identical geometrical transformation was observed in **4**, with the piperidine ring at position 5, while the other moiety at position 2 remained approximately coplanar to the central conjugation plane. However, no interstate crossing was noticed during the optimization, possibly because of the mild geometry changes and the dominating influence of the amino group at position 2.

## 3.3. pH sensing properties

Benzimidazole and quinoline derivatives are often considered as functional moieties in design and development of novel fluorescent sensors [10]. Binding of a proton, metal ions or various anions affects the extent of *π*-conjugation and changes the electron distribution within the aromatic fragments. Thus, it is expected that the protonation of such molecular systems would cause a change in the emission intensity and colour, which is the main precondition for the development of novel fluorescent pH probes and sensors.

Structure of amino-substituted derivatives of benzimidazo[1,2-*a*]quinoline-6-carbonitrile **1**–**10** is potentially very attractive for development of fluorescent sensors for the determination of pH over a wide pH scale. Several ionisable positions in molecules enable either protonation or deprotonation accompanied by pronounced spectral (colour) changes that are visible by the naked eye.

pH sensing properties of the selected benzimidazo[1,2-*a*]quinoline-6-carbonitriles were investigated in buffer solutions (*v*/*v* EtOH:H2O = 1:99), ethanol (*v*/*v* EtOH:H2O = 99:1) and mixed solutions (*v*/*v* EtOH:H2O = 50:50). The observed trends in spectroscopic properties upon protonation, and spectral shifts caused by changing pH are in good agreement for all examined solvent mixtures. In scope of immobilisation and further examination of **1**–**10** in plasticised polymer matrices, we selected ethanol as the most suitable solvent for the comparison of spectral properties. Thus, focus of this work was placed on the detailed characterization of spectral properties in ethanol and 50:50 mixtures, while aqueous solutions were used for the initial p*K*a determination and to gain insight into structural effects in this class of systems that determine acidity/basicity constants in this common solvent.

Typical spectral changes observed due to protonation are shown in **Figure 5**. Absorbance and fluorescence data for neutral and protonated forms of the investigated compounds in ethanol are presented in **Table 2**.



**Figure 5.** Effect of pH on A) absorption (*c* = 1×10–5M) and B) fluorescence (*c* = 1×10–7M) spectra of **2** in H2O/ethanol solution (*v*/*v* : 50/50), *λ*exc = 433 nm. Colour change from yellow to orange and a ~10-fold decrease in the fluorescence intensity upon protonation is observed for **2** inH2O/ethanol solvent mixture (*v*/*v* : 50/50).

**Table 2.** Effect of pH on the spectral properties of selected compounds. Absorbance and fluorescence data were collected in acidified ethanol (*v*/*v* 99:1 EtOH:H2O) and pure ethanol solvent. Experimental and calculated p*K*a values are given for aqueous solution.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **1** | | **2** | | **3** | | **4** | | **6** | | **8** | | **9** | | **10** | |
|  | **pH=1.5** | **pH=7** | **pH=1.5** | **pH=7** | **pH=1.5** | **pH=7** | **pH=1.5** | **pH=7** | **pH=1.5** | **pH=7** | **pH=1.5** | **pH=7** | **pH=1.5** | **pH=7** | **pH=1.5** | **pH=7** |
| ****abs (nm)** | 262  347 | 348 | 450 | 433 | 380 | 369 | 369  408 | 397  420 | 450 | 410  433 | 425 | 407  426 | 353 | 353 | 342  357 | 344  360 |
| ***ε* x 103 (dm3mol-1cm-1)** | 29.55  9.63 | 7.89 | 36.02 | 40.82 | 18.32 | 12.07 | 20.32  21.56 | 20.88  19.02 | 31.48 | 26.22  31.91 | 17.43 | 18.20 | 14.42 | 6.91 | 13.62  14.64 | 10.74  12.00 |
| ****emiss (nm)** | 469 | 469 | 468 | 462 | 405 | 405 | 493 | 463 | 464 | 458 | 478 | 450 | 483 | 480 | 488 | 503 |
| **Rel. Fluo. Int.a** | 365.54 | 372.70 | 23.68 | 411.18 | 278.01 | 182.67 | 56.89 | 253.12 | 31.71 | 659.69 | 187.20 | 319.81 | 780.36 | 827.22 | 389.17 | 44.58 |
| **p*K*a solutionb** | no spectral change | | 2.5 | | 4.0 | | 3.2 | | 2.8 | | ≈ 2.5  ≈ 8.5 | | <1.7 | | 2.3 | |
| **p*K*a calculatedc** | 3.2IM | | 2.72-PIP  1.5IM | | 4.5IM  –6.05-PIP | | 6.4IM  1.12-PIP  –6.55-PIP | | - | | - | | - | | - | |

**a** Relative fluorescence intensities are given to compare fluorescence intensity properties of protonated and neutral form of each compound. Due to differences in quantum yields, data are collected at different slit width for each compound.

**b**Experimental p*K*a values aredetermined from the absorbance data collected in aqueous solution *v/v* 99:1H2O: EtOH.

**c**Different computational p*K*a values correspond to successive protonation equilibria, with subscript IM indicating the ring imino nitrogen, while subscripts 2-PIP and 5-PIP denote the piperidine nitrogen at positions 2 and 5 as the protonation sites, respectively.

Aminated benzimidazo[1,2-*a*]quinoline-6-carbonitriles **1**–**10** in alkaline solution (pH = 10) exhibit absorption maxima between 420 nm and 435 nm. According to the computational results (Section 3.2.), at pH = 10 these systems are present as neutral unionized compounds. Upon lowering the pH conditions, most of the investigated dyes get protonated [13, 31]. Protonation of the imine nitrogen enhances polarization in **2**, **3**, **6** and **7** causing batochromic shift (10 – 17 nm) of the monocation absorption band. Colour change from yellow to orange can be observed visually. However, upon acidification of **4**, **5** and **8**, the absorption band was blue shifted and visually observed as the colour change from yellow to colourless. It is attributed to the subsequent protonation at the other nitrogen atoms in a molecule, causing blue shift as the protonated lone pair electrons become no longer involved in the *π*-conjugation.

Changes in the fluorescence spectra of **1**–**10** upon protonationare caused by intramolecular donor-acceptor interactions. Upon acidification, the *push - pull* polarization determine spectral response in all aminated fluorescent dyes. The emission band is batochromicaly shifted and fluorescence is quenched upon protonation in most of the investigated compounds. A significant change in the fluorescence intensity upon protonation enables development of novel pH sensing molecular systems and a step toward fluorescent sensing materials.

The p*K*a values were experimentally determined from spectroscopic titration data in aqueous solution (*v*/*v* 99:1 H2O:EtOH). For the first protonation, these range from p*K*a = 1.7 for **9**, to p*K*a = 4.0 for **3** intheaqueous solution. Compound **1** did not reveal spectral changes under investigated conditions (pH = 1–13), leading to a conclusion that it is a weaker base than other investigated systems. According to the obtained results, examined fluorophores can be used as pH sensing molecules in the acidic media. However, the apparent p*K*a values can be drastically modified by changing the molecule environment, such as immobilisation in polymer matrices, as it will be revealed later.

The compounds **1**–**10** can undergo a stepwise protonation at nitrogen atoms within each molecule, according to the general schematic representation of aminated benzimidazo[1,2-*a*]quinoline-6-carbonitrile acid-base equilibria at **Scheme 1.**

**Scheme 1**. General acid-base equilibrium of the aminated benzimidazo[1,2-*a*]quinoline-6-carbonitriles.

Study of acid-base properties and determination of the dominant protonation forms at different pH values in water was conducted by computations, which focused on derivatives 1–4 since these systems showed the highest application potential.

Calculated aqueous p*K*a values for **1**–**4** are presented in **Table 2**. Validity of the computational approach was probed by calculating the p*K*a values for model systems, including benzimidazole, imidazole and piperidine. The obtained values of 5.0, 6.1 and 11.2, respectively, were found in excellent agreement with experimentally determined 5.6, 6.95 and 11.2 [45], in the same order, which lends credence to the applied computational methodology and renders other theoretical results as reliable.

System **1** gets protonated on the ring imino nitrogen and this process is associated with the p*K*a value of 3.2, which reveals it modest basicity. This makes it less basic than benzimidazole (p*K*a = 5.6), but also than the analogous derivative without the -CN group, for which our calculations give p*K*a = 6.4. This indicates that the basicity of **1**, and its derivatives **2**–**3** (see later), is affected by the incorporation of the benzimidazole core within a larger conjugated aromatic framework, and even more by the presence of the electron-withdrawing -CN group, that is known to reduce the basicity [46-48]. Addition of the electron-donating piperidine moiety changes the site of the protonation. As piperidine is intrinsically more basic than benzimidazole, it becomes the site of the first proton attachment in **2**, while, interestingly, this remains the imino nitrogen in **3**. It turns out that in **2**, the piperidine moiety is positioned far away from the electron-withdrawing -CN group, which favours the protonation at its amino moiety. The resulting p*K*a is lower at 2.7, in excellent agreement with the p*K*a = 2.5 measured here. In **3**, piperidine is placed in the vicinity of the -CN group, which reverts the protonation site back to the ring imino nitrogen. It benefits from the electron-donating effect of the nearby piperidine, thus leading to the enhanced basicity of p*K*a = 4.5. This is confirmed by the corresponding C(ring)–N(piperidine) bond distance of 1.381 Å in **3**, which is significantly shortened in protonated **3+** to 1.360 Å, thus indicating the participation of the piperidine nitrogen in the resonance stabilization of the excess positive charge. The second protonation in **2** is associated with p*K*a = 1.5, being too low to have any notable impact in the measured UV/Vis spectra under investigated conditions, while in **3** it is even lower at p*K*a = –6.0, as our calculations show.

Basicity of **4** is a result of interplay of effects revealed in **2** and **3**. The proton is most favourably attached to the ring imino nitrogen, while both piperidine units participate in the cationic resonance. The corresponding C(ring)–N(piperidine) distances are shortened from 1.385 and 1.392 Å in **4** to 1.367 and 1.379 Å in **4+**, thus confirming our conclusion. This increases basicity to p*K*a = 6.4, which seemingly differs from the experimentally determined apparent value of 3.2, yet both the experimental and calculated values consistently indicate its increased basicity relative to **1**–**3**. The second protonation to **42+** occurs on the piperidine nitrogen at position 2 with p*K*a = 1.1, which is even lower than in **22+**, and also less significant, while the third protonation to **43+** requires a very strong acid below p*K*a = –6.5.

## 3.4. Immobilisation of fluorescent pH sensing molecules

Very bright and pH responsive fluorescence of benzimidazo[1,2-*a*]quinoline-6-carbonitriles suggested their use as novel pH sensitive chromoionophores in fluorescent ion-selective optodes [49]. For that purpose the most promising candidates amongst **1**-**10** were identified and immobilised in thin polymer matrices.

Immobilisation of the selected compounds in DOS plasticised PVC matrices resulted in homogenous yellow (**2**, **3**, **6**, **10**) or colourless (**1**, **9**) thin polymer films, which exhibit strong blue emission (**Figure 6**) observed under UV lamp (*λ*exc = 366 nm). Absorbance and fluorescence emission data of the immobilised compounds in thin polymer films are presented in **Table 3**.

**Table 3.** Absorption and fluorescence emission data of immobilised compounds in thin polymer films, *c* = 1x10–3 M.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Compound** | ****abs (nm)** | ****emiss (nm)** | **Rel. Fluo. Int.** | **p*K*a film** |
| **1** | 350 | 388  455 | 282.02  192.35 | no spectral change |
| **2** | 415  435 | 465  485 (sh) | 349.14 | 5.5 |
| **6** | 417  440 | 460  480 (sh) | 299.93 | 5.7 |
| **9** | 353 | 388  467 | 199.16  224.59 | no spectral change |
| **10** | 357 | 388  498 | 158.80  86.26 | ≈2.8 |

Selected fluorescent dyes reveal spectral characteristics in thin films of similar properties to those in ethanol, however with slight bathochromic shifts (Δ*λ* = 3 – 5 nm). In general, immobilised compounds exhibit a naked-eye observable colour change upon protonation (yellow to orange) and absorbance shift towards higher wavelengths.

Absorbance and emission changes due to protonation of immobilised **2** are shown in **Figures 6A** and **6B**.



**Figure 6**. Effect of pH on A) absorption and B) fluorescence spectra of compound 2 immobilised in plasticised PVC films. Absorbance (**abs = 460 nm) (C) and fluorescence (**emiss = 500 nm) (D) *vs*. pH. The inset shows the range between pH 4 and 8, in which the response is quasi-linear.

The relationship between the absorbance, *A*, and the logarithm of the hydrogen concentration is sigmoidal, exhibiting quasi-linear response between pH 4 and 8 (**Figure 6C** and **6D**). Data points were fitted to a Boltzmann function and typical calibration curve is presented in **Figure 7**. The curve shows excellent adjustment (R2 = 0.9981).



**Figure 7.** A) Photographs of thin film observed by daylight and under UV lamp. B) Absorbance versus pH of **2** (**abs = 460 nm), in ethanol solution and immobilised in a thin film. A Boltzmann curve, which can be used as a calibration function, was fit to the experimental data.

The sensing range of the studied dyes is determined by the acid-base equilibrium. However, typical response of sensing membranes is not dependent solely on the H+ concentration. There are several competing reactions which may occur in thin polymer films. Co-extraction of anions from the solution into the membrane is the most common cause of the interfering reactions, which is especially obvious at higher concentrations of buffer solutions. Therefore, acid-base properties are described as “apparent” p*K*a values. p*K*a apparent values corresponding to the calibration curves and absorbance and emission maxima of immobilised dyes are shown in **Table 3**.

The obtained results indicate that general sensing properties of chromophores have improved upon immobilisation in the plasticised films. An important finding is that the immobilisation of compounds led to an increase in their apparent p*K*a values by around 2–4 units. For example, the immobilisation of **2** in the plasticised PVC matrix changed the apparent p*K*a value from p*K*a apparent = 1.8 in ethanol (p*K*a determined in water = 2.5) to p*K*a apparent = 5.5 (**Figure 7**). Such effects are expected in polymer films where a number of parameters, including lipophilicity, polarity and microviscosity affect the complex heterogeneous ion-exchange equilibria. Nevertheless, the observed p*K*a shift towards neutrality promotes the investigated class of fluorophores as promising candidates for use as a fluorescent chromoionophores in ion-exchange optodes.

Prepared pH sensing membranes exhibit reversible response, as presented in **Figure 8**. Response time of investigated pH sensing membrane is 17 sec (Figure S10).



**Figure 8.** Reversible pH response of **2** (protonated – neutral) at selected absorbance (*λ* = 460

nm) and emission wavelength *λ* = 500 nm), *λ*exc = 460 nm.

## Conclusions

Systematic evaluation of a class of 2-amino substituted benzimidazo[1,2‑*a*]quinoline-6-carbonitrile dyes as novel fluorescent pH probes and pH sensing materials or H+ fluoroionophores in bulk optode membranes was conducted. It can be concluded that intramolecular interactions of the examined compounds, and consequently photophysical properties of examined dyes, are significantly affected by the position, type or length of amino substituents and the observable trends can be singled out. Detailed spectral characterisation and structure-property study revealed that investigated class of molecules exhibit bright fluorescence in blue-green part of spectrum with clear emission maxima between 480–500 nm, while the calculations were utilized to understand the substituent effects governing the electronic transitions responsible for the observed spectra. The latter also revealed that lowest electron excitations in all systems have the *π*–*π*\* character, while the lowest excited states and the frontier molecular orbitals show a high degree of similarity systems, arising from the common benzimidazo[1,2-*a*]quinoline skeleton. The expected *push-pull* interactions between the introduced amino and nitrile groups are evident in **2** and **4**, and less discernible in **3** due to the less favourable orientation of its quinoline ring at position 5 as a result of the steric repulsion with the nearby atoms.

Calculated aqueous p*K*a values indicate that molecules **1**-**4** are unionized at neutral pH, while the corresponding transition to monocationic forms occurs at acidic pH values between 2.7 – 6.4. The obtained results also hint at a possibility that at highly acidic conditions around and below pH = 1, dicationic species could occur with **2** and **4**, but this is not reflected as significant changes in the observed spectra under such environment. Electron-donating amino substituents are beneficial for the basicity, particularly when placed at the position 5 of the tetraaromatic skeleton, where they are able to diminish basicity-reducing effect of the -CN moieties.

Application of presented class of compounds as pH sensing materials is demonstrated by immobilisation in plasticized polymer matrix. Bright and blue emissive thin films showed pH response and dynamic range around p*K*a = 6, making it suitable for a wide range of applications, for example as fluoroionophores in ion-selective optodes.

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