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Highly Photostable Near-Infrared Fluorescent pH Indicators and Sensors Based on BF$_2$-Chelated Tetraarylazadipyrromethene Dyes

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ABSTRACT: In this study, a series of new BF$_2$-chelated tetraarylazadipyrromethane dyes are synthesized and are shown to be suitable for the preparation of on/off photoinduced electron transfer modulated fluorescent sensors. The new indicators are noncovalently entrapped in polyurethane hydrogel D4 and feature absorption maxima in the range 660–710 nm and fluorescence emission maxima at 680–740 nm. Indicators have high molar absorption coefficients of $\sim$80 000 M$^{-1}$ cm$^{-1}$, good quantum yields (up to 20%), excellent photostability and low cross-sensitivity to the ionic strength. $p_K_a$ values of indicators are determined from absorbance and fluorescence measurements and range from 7 to 11, depending on the substitution pattern of electron-donating and -withdrawing functionalities. Therefore, the new indicators are suitable for exploitation and adaptation in a diverse range of analytical applications. Apparent $p_K_a$ values in sensor films derived from fluorescence data show 0.5–1 pH units lower values in comparison with those derived from the absorption data due to Förster resonance energy transfer from protonated to deprotonated form. A dual-lifetime referenced sensor is prepared, and application for monitoring of pH in corals is demonstrated.

Design, synthesis, and spectroscopic/photophysical characterization of novel fluorochrome chemosensors remains a central research field in analytical chemistry.¹ The measurement of pH by fluorescence-based techniques is well established for both imaging and sensing applications²,³ in various fields of experimental science, such as biomedical research,⁴–¹⁰ marine biology,¹¹,¹² and biotechnology.¹³,¹⁴ The most frequently used fluorescent pH indicators are 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS), carboxyfluorescein derivatives, seminaphthorhodamines (SNARFs), and hydroxycoumarins.¹⁵–¹⁹ However, these indicators suffer from several drawbacks. For example, carboxyfluorescein has only moderate photostability, and the photostability of 2′,7′-dihexylfluorescein (suitable for measurements in seawater) is very poor;²⁰ the $p_K_a$ value of HPTS is highly dependent on ionic strength of solution; and most coumarins are excitable only by high-energy radiation in the range from 350 to 450 nm. In biological applications, it is desirable to use fluorophores with absorption/emission profiles in the red or near-infrared (NIR) spectral regions because they have many advantages: significant reduction of the background signal due to low absorption and autofluorescence of biomolecules in the NIR region, low light scattering and deep penetration of the NIR light, and the possibility to use low-cost excitation light sources. Despite the optical benefits, there is a surprising scarcity of pH indicators that have such desired absorption and emission properties. Although the water-soluble SNARF indicators and their lipophilic derivatives do absorb at $\sim$630 nm, they possess only moderate brightness and photostability.²¹ The same holds for the cyanine dyes, which are well-known NIR chromophores that have only scarcely been proposed as pH indicators.²²

In contrast, BF$_2$-chelated tetraarylazadipyrromethane dyes (aza-BODIPYs) represent an interesting class of NIR chromophores that are amenable to structural modification and exhibit excellent photophysical properties.²³ Several fluorescent pH indicators based on aza-BODIPY dyes bearing amino- or hydroxy-functionalized substituents were reported by O’Shea and co-workers.²⁵–²⁷ These on/off pH indicators show photoinduced electron transfer from an amino group or a twisted phenolate to the aza-BODIPY subunit, causing fluorescence quenching in the deprotonated state. However,
these probes cover only acidic and near-neutral range. A systematic study of the properties of these pH indicators and possible synthetic modifications with respect to tuning the pKₐ values has not previously been reported in the literature.

In the present study, we investigated the synthesis and characterization ofaza-BODIPY fluorophores that can probe pH changes by large associated changes in their emission intensity around 700 nm and possess pKₐ values in the physiological and alkaline pH range. Eight 4,4'-difluoro-4-bora-3a,4a-triaza-s-Indacene dyes that can detect pH through a photoinduced electron transfer process were synthesized and characterized. We show that the pKₐ values of the new indicators can be tuned over a wide range, and simple prediction rules can be derived. This enables a variety of applications for sensors and imaging. As an example, an application of the new sensing materials for fiber-optic pH measurements in marine biology is demonstrated.

**EXPERIMENTAL SECTION**

**Materials.** 3'-Chloro-4'-hydroxyacetophenone, 1,3-diphenyl-2-propenone, tera-tert-butyl-29H,31H-phtalocyanine, N,N-disopropylethylamine, ammonium acetate, benzaldehyde, semiphenanthrohodafuor decyl ester (SNARF-DE, chromoionophore XIII), boron trifluoride diethyl etherate, MOPS buffer salt, and anhydrous sodium sulfate were purchased from Sigma Aldrich (www.sigmaaldrich.com). 3'-Hydroxyacetophenone, 4'-hydroxylchalcone, 4'-methoxylchalcone, 4,4'-dimethoxylchalcone, and nitromethane were obtained from ABR (www.abcr.com). 4'-Hydroxy-3'-methylacetophenone was obtained from TCI Europe (www.tci-europe.de). Deuterated dimethyl sulfoxide was obtained from Euriso-top (www.eurisotop.com). All other solvents (synthesis grade) as well as sodium chloride and the bufluoride diethyl etherate, MOPS buffer salt, and anhydrous sodium sulfate were purchased from Sigma Aldrich (www.sigmaaldrich.com). Polyethylene glycol terephthalate (Mylar) was obtained from Goodfellow (www.goodfellow.com). Microcrystalline powder of phosphor chromium(III)-activated gadolinium aluminium borate (Gd₅Al₄.75Cr0.25O₁₂, Cr-GAB) was prepared similarly from 0.1 mg of indicator dye Ar-gated silver CD (400 mg) with addition of 200 mg of Cr-GAB particles. Subsequently, it was coated on the tip of an optical fiber (Ø 400 μm) from Specialty Photonics (www.specialtyphotronics.com). The sensors were allowed to dry 30 min in the ambient to ensure complete evaporation of the solvent. Between measurements, the sensors were stored in darkness at room temperature.

**Methods.** Absorption measurements were performed on a Cary 50 UV–vis spectrophotometer from Varian (www.varianinc.com). The molar absorption coefficients were determined as an average of three independent measurements for the concentrations of the dyes 2.5–3.4 × 10⁻⁶ M. Fluorescence spectra were recorded on a Hitachi F-7000 spectrofluorometer (www.hitachi.com). Relative fluorescence quantum yields were determined according to Demas and Crosby²⁵ using tera-tert-butyl-29H,31H-phtalocyanine (Fluka, www.sigmaaldrich.com) as a standard (quantum yield = 0.44).³⁰ Two independent measurements were performed to obtain the average value. The concentration of the indicators was kept below 1.5 × 10⁻⁶ M to avoid dye aggregation and reabsorption of the fluorescence. NMR spectra were recorded.

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on a 300 MHz instrument (Bruker) in DMSO- $d_6$ or CDCl$_3$ with TMS as a standard. Electron impact (EI, 70 eV) mass spectra were recorded on a Waters GCT Premier equipped with direct insertion (DI). The pH of the buffer solutions (CHES, MES, and CAPS) was controlled by a digital pH meter (InoLab pH/ion, WTW GmbH & Co. KG, www.wtw.com) calibrated at 25 °C with standard buffers of pH 7.0 and pH 4.0 (WTW GmbH & Co. KG, www.wtw.com). The buffers were adjusted to constant ionic strength (IS = 0.02 or 0.15 M) using sodium chloride as the background electrolyte. Dual-lifetime referenced measurements were performed with a fiber-optic Firesting fluorometer from Pyroscience (www.pyro-science.com) with a modulation frequency of 4 kHz.

Photobleaching experiments in solutions were performed by irradiating the samples with light from a 642-nm high-power 10 W LED array (www.led-tech.de) focused through a lens purchased from Edmund optics (www.edmundoptics.de). The photodegradation profiles were obtained by monitoring the absorption spectra. For the leaching test, sensor foil (D4) was placed in a flow-through cell, and the absorption of the films was monitored while aqueous buffer (IS = 0.02 M) was passed through it. A detailed description of the pH measurements in the gastric cavity of a symbiont bearing coral (Goniopora sp.) is contained in the Supporting Information.

**RESULTS AND DISCUSSION**

**Synthesis.** Two general methods to prepare azadipyrromethene chromophore are known. In the first method, 2,4-diarylpyrroles act as precursors, and are converted into their 5-nitroso derivatives, which are then condensed with a second molecule of pyrrole to yield unsymmetric derivatives. In the second method, nitromethane adducts of chalcones are reacted with ammonium salts at elevated temperatures to give symmetric azadipyrromethenes. Conversion of the diarylnitroketones into pyrroles and 5-nitrosopyrroles and the isolation of these products is not required in this method.

Unsymmetrical derivatives of the aza-BODIPYs bearing only one pH-sensitive group were strongly preferable to the symmetrical ones for several reasons: (i) a simple acid–base equilibrium including only two forms of the indicator; (ii) significantly higher hydrophobic character of the monosubstituted derivatives compared to the symmetrical ones, which prevents the dye from leaching into an aqueous environment, particularly in case of the deprotonated form; (iii) a low charge of the deprotonated form (1$^-$), which is expected to minimize the effect of the ionic strength on the sensing properties.

Keeping practical applications in mind (in which accessibility of the indicators is very important) we decided to employ the second method for preparation of the unsymmetrical dyes. In this approach, condensation of two different nitrochalcones results in a mixture of the aimed unsymmetrical aza-BODIPY and two symmetrical derivatives (Scheme 1), which are easily separated via chromatography on silica gel. The starting compounds for the synthesis were the diaryl $\alpha,\beta$-unsaturated ketones (chalcones) that are either commercially available (for the synthesis of 1, 5, 7, and 8) or prepared by Claisen–Schmidt condensation (for the synthesis of 2–4, 6). These were synthesized from the corresponding aldehyde and acetophenone with KOH as a base, for the synthesis of 1, 5, 7, and 8) or prepared by Claisen–Schmidt condensation (for the synthesis of 2–4, 6). These were synthesized from the corresponding aldehyde and acetophenone with KOH as a base, yields the 1,3-diaryl-4-nitrobutan-1-ones in essentially quantitative yields after
Ar4 rings with the aza-BODIPY chromophore and the absence of any substituents (650 nm). The absorption of the probes bearing a hydroxyl group in the p-position (R1) of Ar4 is rather similar (668–714 nm). The absorption maxima of indicators in hydrogel D4 mirrored closely that of the parent compound (677 and 714 nm, respectively). The emission of the probes bearing a hydroxyl group in the p-position (R1) of Ar4 was located between 699 and 714 nm. The compounds containing OH or CH3 in the p-position of the Ar2 and Ar4 rings result in the bathochromic shift of the absorption spectrum but have no effect on the spectral properties if located in the Ar1 and Ar3 rings.

**Photophysical Properties.** The new aza-BODIPY compounds were dissolved in an EtOH/aqueous buffer mixture (1:1), and their spectroscopic properties were investigated (Figure S1 of the Supporting Information, Table 1). The absorption of the probes bearing a hydroxyl group in the p-position (R1) of Ar4 is rather similar (668–677 nm). The shortest wavelengths of the absorption maxima are observed for 5 and 6 (656 and 650 nm, respectively). These values are very close to the tetraphenyl-substituted aza-BODIPY chromophore lacking any substituents (650 nm).32 The absorption of 7 (bearing a methoxy group in the p-position of the Ar3 ring) is bathochromically shifted ~20 nm as compared with 1. On the other hand, introduction of the second methoxy group in the p-position of the Ar2 ring (8) results in even minor changes compared with 7.

These data indicate pronounced conjugation of the Ar3 and Ar5 rings with the aza-BODIPY chromophore and the absence of such conjugation for the Ar1 and Ar2 rings. This conjugation can be caused by a hydrogen bond between the fluorine atom and the hydrogen atom located in the o-position of Ar3 and Ar5 rings. Evidently, the electron-donating substituents (–OH or O–CH3) in the p-position of the Ar2 and Ar4 rings result in the bathochromic shift of the absorption spectrum but have no effect on the spectral properties if located in the Ar1 and Ar3 rings. As expected, the hydroxyl group in the m-position of the Ar3 has virtually no effect on the spectral properties. The molar absorption coefficients for all aza-BODIPY derivatives are in the 70 000–90 000 M−1 cm−1 range.

It should be mentioned that precision of determination of molar absorptivities as well as fluorescence quantum yields can be affected by impurities of the probes. Although all the indicators were obtained as single crystals, several indicators show solvent impurities in the 1H NMR spectrum. As revealed by crystallographic study of 8 (Figure S2 of the Supporting Information), this is explained by incorporation of solvent molecules (tetrahydrofuran and hexane) into the crystal structure. Evidently, this slightly affects the calculated molar absorption coefficients. For instance, the crystals of 8 contain one molecule of tetrahydrofuran per each molecule of the dye, and this results in the reduction of molar absorption coefficients by about 8.8%.

The trend observed in absorption maxima was the same for fluorescence emission maxima (Table 1). The emission maxima of the probes bearing a hydroxyl group in the p-position (R1) of Ar4 were located between 699 and 714 nm. The compounds 5 and 6 clearly showed a hypsochromic shift (676 and 691 nm, respectively), and the emission of 7 and 8 was shifted bathochromically (721 and 722 nm, respectively) as compared with the parent compound 1. The trends in the absorption and fluorescence maxima of indicators in hydrogel D4 mirrored aqueous workup, which were then used without further purification. Condensation with ammonium acetate in refluxing butanol gave the azadipyrromethenes via a cascade of events (in situ formation of the pyrrole and corresponding nitrosopyrrole and subsequent condensation of those two entities). The obtained mixture was purified by chromatography with hexane/dichloromethane in the case of 1–6 or toluene/dichloromethane in case of 7 and 8. Finally, complexation of the azadipyrromethenes with boron trifluoride gave the aza-BODIPYs in good yields.

**Table 1. Photophysical Properties of the aza-BODIPY Probes: Absorbance Maxima for the Acidic (λ_{abs-acid}) and the Basic Forms (λ_{abs-base}), Emission Maxima for the Acidic Form (λ_{em-acid}), Molar Absorption Coefficients (ε), and Luminescence Quantum Yields (QY)**

<table>
<thead>
<tr>
<th>dye</th>
<th>λ_{abs-acid}/λ_{abs-base} (EtOH/H2O-1:1) (nm)</th>
<th>λ_{em-acid} (EtOH/H2O-1:1) (nm)</th>
<th>λ_{abs-acid}/λ_{abs-base} (hydrogel D4) (nm)</th>
<th>λ_{em-acid} (hydrogel D4) (nm)</th>
<th>ε ± 5% (M⁻¹ cm⁻¹)</th>
<th>QY ± 20% (EtOH/buffer-1:1)</th>
<th>QY ± 20% (THF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>670/726</td>
<td>702</td>
<td>687/742</td>
<td>718</td>
<td>84 000</td>
<td>0.15</td>
<td>0.11</td>
</tr>
<tr>
<td>2</td>
<td>668/734</td>
<td>699</td>
<td>683/754</td>
<td>730</td>
<td>80 600</td>
<td>0.16</td>
<td>0.08</td>
</tr>
<tr>
<td>3</td>
<td>675/735</td>
<td>709</td>
<td>692/750</td>
<td>720</td>
<td>86 200</td>
<td>0.18</td>
<td>0.08</td>
</tr>
<tr>
<td>4</td>
<td>677/741</td>
<td>714</td>
<td>694/752</td>
<td>722</td>
<td>89 700</td>
<td>0.16</td>
<td>0.05</td>
</tr>
<tr>
<td>5</td>
<td>656/782, 605</td>
<td>691</td>
<td>672/859, 615</td>
<td>702</td>
<td>69 500</td>
<td>0.07</td>
<td>0.03</td>
</tr>
<tr>
<td>6</td>
<td>650/656</td>
<td>676</td>
<td>660/663</td>
<td>686</td>
<td>71 100</td>
<td>0.10</td>
<td>0.02</td>
</tr>
<tr>
<td>7</td>
<td>687/743</td>
<td>722</td>
<td>707/760</td>
<td>736</td>
<td>80 100</td>
<td>0.17</td>
<td>0.14</td>
</tr>
<tr>
<td>8</td>
<td>690/750</td>
<td>721</td>
<td>708/768</td>
<td>736</td>
<td>74 000</td>
<td>0.16</td>
<td>0.11</td>
</tr>
</tbody>
</table>

For the protonated form.
those in solution with an additional bathochromic shift of ∼15 nm (Table 1).

The fluorescence quantum yields (QYs) of most indicators were very similar (0.14–0.17 in tetrahydrofuran, Table 1). Again, the dyes 5 and 6 represent a notable exception as they exhibited significantly lower quantum yields. As can be seen, all the values are lower in EtOH/aqueous buffer. Although very low concentrations of the dyes were used (<1.5 × 10−4 M), some aggregation of the dyes in EtOH/water mixture cannot be excluded. On the other hand, lower quantum yields in this media can be explained by more efficient radiationless deactivation involving O–H vibrations. It should be mentioned that the QYs of most reported NIR-emitting dyes are generally lower than for those emitting in the visible part of the spectrum.

In summary, the photophysical properties of the new aza-BODIPY derivatives retain most of the advantages of the aza-BODIPY fluorophores (except for 5 and 6), including narrow bandwidth, high molar absorption coefficient, and acceptable fluorescence quantum yields.

**pH-Sensing Properties.** pKₐ values of the new probes were determined both in ethanol/aqueous buffered solution (1:1) and in a hydrogel D4 film from the absorption measurements. As can be seen, the absorption spectra shift bathochromically upon deprotonation of the hydroxyl group in all cases except for 6 as a result of the absence of conjugation with the aza-BODIPY core (Figure 1, Table 1). Notably, the absorption spectrum of deprotonated 5 is rather unusual and shows two peaks (Figure 1b). The pKₐ values determined at two ionic strengths of the solution (0.02 and 0.15 M) were very similar (Table 2). Such low cross-sensitivity to the ionic strength is unexpected, possibly because of different media used by Murtagh et al. for the calibration of the pH indicator (nonionic surfactant) and higher concentration of the dye in these micelles. As will be demonstrated in the following, the concentration of the indicators is rather critical for determination of apparent pKₐ values via fluorescence measurements.

The fluorescence of the indicators is quenched upon deprotonation (Figure 2), which is attributed to efficient photoinduced electron transfer. In fact, no emission is detected for the deprotonated form of the dyes. The apparent pKₐ values (pKₐ) obtained from the emission measurements in solution are very close to those determined from the absorption spectra (Table 2).

![Figure 2](dx.doi.org/10.1021/ac3011796/s1/1.png)

**Table 2. pKₐ Values of Fluorescent aza-BODIPY Probes**

<table>
<thead>
<tr>
<th>dye no.</th>
<th>pKₐ (IS = 0.02M) (EtOH/H₂O 1:1)</th>
<th>pKₐ (IS = 0.02M) (EtOH/H₂O 1:1)</th>
<th>pKₐ (IS = 0.15M) (EtOH/H₂O 1:1)</th>
<th>pKₐ (IS = 0.02M) (hydrogel D4)</th>
<th>pKₐ (IS = 0.02M) (hydrogel D4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.35</td>
<td>8.38</td>
<td>8.36</td>
<td>8.41</td>
<td>8.47</td>
</tr>
<tr>
<td>2</td>
<td>7.00</td>
<td>7.01</td>
<td>6.92</td>
<td>6.98</td>
<td>6.73</td>
</tr>
<tr>
<td>3</td>
<td>8.36</td>
<td>8.47</td>
<td>8.42</td>
<td>8.45</td>
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<tr>
<td>4</td>
<td>8.06</td>
<td>8.05</td>
<td>8.11</td>
<td>8.15</td>
<td>7.94</td>
</tr>
<tr>
<td>5</td>
<td>9.69</td>
<td>9.68</td>
<td>9.69</td>
<td>10.88</td>
<td>9.84</td>
</tr>
<tr>
<td>6</td>
<td>11.05</td>
<td>10.88</td>
<td>11.02</td>
<td>9.69</td>
<td>12.35</td>
</tr>
<tr>
<td>7</td>
<td>8.71</td>
<td>8.65</td>
<td>8.76</td>
<td>8.74</td>
<td>8.81</td>
</tr>
<tr>
<td>8</td>
<td>8.92</td>
<td>8.89</td>
<td>8.90</td>
<td>8.94</td>
<td>9.11</td>
</tr>
</tbody>
</table>

The pKₐ of the aza-BODIPY derivatives can be tuned over a wide range by introducing electron-withdrawing/donating (remote) neighboring functionalities or changing the position of the hydroxyl group. If probe 1 (with pKₐ of 8.38) is substituted at the m-position with a chlorine atom, the pKₐ drops to 7.01 (2). When (inductively) electron-donating methyl groups are introduced in the m-positions, the pKₐ is 8.47 for one methyl group (3) and 8.05 for two methyl groups (4). The increased acidity of a dimethyl-substituted derivative is surprising, but can be explained by the difficulties of formation of the solvent adduct due to steric hindrances. If a hydroxyl group is introduced in the p-position of the Ar² aryl ring instead of the Ar¹ aryl ring, the pKₐ increases to 9.68 (5). This is very close to the pKₐ value of phenol (pKₐ = 10), which indicates the absence of the conjugation with the aza-BODIPY core. This observation is in good agreement with the trends obtained from the absorption spectra. A drastically high pKₐ of 6 (10.88) compared with the pKₐ of parent compound 1 (8.38) was attributed to the formation of an intramolecular hydrogen bond between the hydroxyl group and the neighboring fluorine atom. Introduction of a remote electron-donating substituent (methoxy group) in the p-position of the Ar³ ring (7) slightly increases the pKₐ value (by about 0.3 units), relative to the parent compound 1. Evidently, the second methoxy group in the p-position on the Ar¹ aryl ring (8) has less effect on the pKₐ value. Effects observed in this study are in line with the electron-withdrawing (or -donating) power of the neighboring (remote) substituents. In summary, pH indicators with tailored pKₐ values can be realized, and the indicators can be easily adapted to particular applications.

The pKₐ values determined in this study differ significantly from the reported pKₐ of 6.9 of a phenolic derivative with a hydroxyl group in the p-position of the Ar¹ ring (similar to the compound 1 in our study) possibly because of different media used by Murtagh et al. for the calibration of the pH indicator (nonionic surfactant) and higher concentration of the dye in these micelles. As will be demonstrated in the following, the concentration of the indicators is rather critical for determination of apparent pKₐ values via fluorescence measurements.

Optical pH sensors were prepared by noncovalent entrapment of the indicators in the hydrogel D4 matrix, which is a highly proton-permeable, uncharged polyurethane derivative with a water uptake capacity of about 50%. The pKₐ of 6.9 of a phenolic derivative with a hydroxyl group in the p-position of the Ar¹ ring (similar to the compound 1 in our study) possibly because of different media used by Murtagh et al. for the calibration of the pH indicator (nonionic surfactant) and higher concentration of the dye in these micelles. As will be demonstrated in the following, the concentration of the indicators is rather critical for determination of apparent pKₐ values via fluorescence measurements.

The fluorescence of the indicators is quenched upon deprotonation (Figure 2), which is attributed to efficient photoinduced electron transfer. In fact, no emission is detected for the deprotonated form of the dyes. The apparent pKₐ values (pKₐ) obtained from the emission measurements in solution are very close to those determined from the absorption spectra (Table 2).
transfer (FRET) from the protonated to the deprotonated form (Figure S3 of the Supporting Information). Considering that FRET is concentration-dependent, this offers another possibility of tuning the dynamic range of the materials. In fact, $pK_a$ values derived from the fluorescence data are lower for higher concentration of the indicators in hydrogel D4 (e.g., $pK_a$ of 6.65, 6.14, 6.03, and 5.89 for the sensor films containing 0.1, 0.25, 0.5 and 1% of the indicator 2), Supporting Information Figure S3c. The $pK_a$ values obtained from the absorption measurements (Supporting Information Figure S10b) are 6.92, 6.83, 7.04, and 7.32 for the same concentrations. This increase may be due to different localization patterns of the dye in hydrogel D4, which is known to be composed of the hydrophilic and hydrophobic regions.

We compared the pH-sensing properties of the complexes and the respective ligands. The ligands were virtually nonfluorescent, possibly due to their nonplanar structure. However, distinct changes in the absorption spectra were observed (Figure S4a of the Supporting Information). The $pK_a$ values were $\sim 1$ unit higher than for the corresponding complexes (Supporting Information Figure S4b). For example, the $pK_a$ values were 8.35 and 7.00 for 1 and 2, but 9.39 and 7.97 for the corresponding ligands. This effect is likely due in part to the lower degree of the π-conjugation between the phenolic substituents and the chromophore core (which is reflected by the smaller shift between the $\lambda_{max}$ of both forms of the dye compared with the corresponding complexes). The electron-withdrawing effect of the BF$_3$ group can also contribute here.

Despite the absence of fluorescence, the nonchelated dyes can be promising as absorption-based indicators.

Dye leaching out of the sensing matrix may be a problem in the case of physically entrapped indicators. It was tested in the case of 1 by monitoring the absorption of the sensing foils. No evident leaching into the aqueous solution was detectable for the protonated form of the dye. In the case of the charged deprotonated form, the decrease in the absorption was very low (0.8% per 24 h, Figure S5 of the Supporting Information), which is within the experimental error. Consequently, leaching is not critical for the investigated sensors due to the pronounced hydrophobicity of the indicator systems.

Photostability. Photostability is a very important parameter for all optical chemosensors. We investigated solutions of the new pH dyes in dimethylformamide under continuous illumination with an ultrabright 642-nm LED array. Figure 3 demonstrates the photodegradation profiles for the new indicators and for seminaphthorhodafluor decyl ester (SNARF-DE), which is used for comparison. The aza-BODIPY probes were significantly more photostable (about 30-fold) than SNARF-DE under identical conditions. In fact, after 2.5 h of illumination, only 1–8% of the dyes was decomposed compared with SNARF-DE, which degrades almost completely after 60 min of irradiation. The photostability trend for aza-BODIPY probes was $6 > 3 > 4 > 1 \sim 2 > 7 > 5 > 8$ (Figure S6 of the Supporting Information). The electron-donating substituents in the aryl rings Ar$^1$ and Ar$^2$ seem to be responsible for decreased photostability; nevertheless, new pH dyes retain excellent photostability of aza-BODIPY dyes, which makes them particularly suitable for long-duration measurements.

Dual-Lifetime Referenced pH Sensor. Fluorescence intensity is a parameter that is easy to measure, but it is influenced by a number of factors, such as the intensity and light field of the excitation source, the sensitivity of the photodetector, and the coloration and turbidity of the measuring media. In contrast, measurements of fluorescence decay time, which is a self-referenced parameter, normally require complicated and expensive equipment. Ratiometric sensing and dual lifetime referencing are popular alternatives. In the latter approach, a referenced material having a long luminescence decay time (typically in the microseconds time domain) is added, and the overall phase shifts are measured in the frequency domain. Since good spectral compatibility (both for excitation and for emission) is essential, a recently reported inorganic phosphor, chromium(III)-doped gadolinium aluminum borate (Cr-GAB)$^{28}$ is an excellent candidate for this purpose; its luminescence decay times of about 100 µs, and high chemical and photochemical inertness make it particularly attractive. The spectral compatibility of the indicator (in its protonated form) and the referenced phosphor is very good (Figure 4) because both are efficiently excited by red LEDs and emit in the same spectral window.

The resulting pH-sensing material is compatible with a commercially available fiber-optic phase oxygen meter (Fire-sting from Pyroscience), which was used for measurements ($\lambda_{exc} \sim 620$ nm). The “sensing chemistry” applied on the tip of a glass optical fiber included 0.1% w/w of indicator 1 and 66% w/w Cr-GAB particles and 33% w/w of hydrogel D4. Different concentrations of indicator and Cr-GAB particles were tested to ensure optimal phase shift dynamics (data not shown). Figure 4c shows a calibration plot of the cotangents of the phase angle, cot $\Phi$ vs pH for the DLR pH-sensing material. The calibration showed an inflection point at pH 8.12, which is very close to the apparent $pK_a$ value obtained from the spectroscopic investigations. Photostability of the indicator at the fiber optic tip was monitored by applying 5 times stronger light intensity and 10 times longer integration times than in the standard settings. For the acidic form, no evident photobleaching was observed after 2000 measurement points, which corresponds to 100 000 measurement points in the standard settings (Figure S7 of ESI). A very small drift of the phase angle (0.2°) was observed in the basic conditions for the same measurement time. Nevertheless, it can be concluded that the fiber-optic sensors possess excellent photostability and can be used for prolonged measurements without recalibration.

Application in Marine Biology. The new pH sensing materials operate in significantly different dynamic ranges and are, therefore, suitable for a variety of important applications. For example, the apparent $pK_a$ values of the indicators 2 and 4 make them particularly suitable for biotechnological and
biological applications, respectively. On the other side, the apparent pK′a of about 8 found for indicator 1 almost ideally matches the pH of the seawater. This new sensing material thus provides a promising alternative to the state-of-the-art optical sensors for seawater pH measurements, which rely on fluorescein derivatives and possess poor photostability. 40,41 We demonstrated this applicability in marine biology by measuring the pH in corals with a fiber-optic pH optode based on the DLR material described above (Figure 5). The pH in corals is affected by both the photosynthetic activity of the algal symbionts in the host tissue, which tend to increase the pH due to their photosynthetic fixation of inorganic carbon, and the respiratory activity of the coral host cells and heterotrophic bacteria associated with the coral, the activity of which tends to decrease pH. Although most pH measurements in corals have focused on the exposed tissues,42 the pH conditions in the gastric cavity (i.e., the coral stomach) are largely unknown; yet, such measurements are highly relevant to understand the microbial processes going on inside corals. Our pH measurements showed a pH gradient of almost 1 pH unit from the coral mouth and into the deepest part of the coral just above the calcium carbonate skeleton (Figure 5b). Evidently, the pH in these parts of the coral was not affected by photosynthetic activity, which was also seen by the absence of any accumulation of O2 (data not shown).

CONCLUSION

In conclusion, we prepared and characterized a series of NIR fluorescent pH indicators with potential to suit a diverse range of analytical applications. pH-sensitive functional aza-BODIPY derivatives were obtained via a simple reaction route starting from commercially available compounds. Except for probes 5 and 6, the new indicators retain the advantages of aza-BODIPY fluorescent probes, such as good brightness and excellent photostability. Variations in both the substitution pattern and the position of hydroxyl functionality allowed manipulation of pK′a values over a wide range, providing valuable information that can be used for future rational design of the indicator systems. The new sensors have high potential for a variety of biotechnological, biological, environmental, etc. applications. As an example, monitoring of pH inside coral polyps was demonstrated. Ongoing work concerns the covalent immobilization of the indicators into the polymeric network, which will completely suppress their leaching, migration, and aggregation and can lead to even better shelf life stability and operating time of the optical sensors.

ASSOCIATED CONTENT

Supporting Information
Details on coral experiments, synthetic procedures, 1H NMR and MS spectra, absorption and emission spectra, calibration curves. This material is available free of charge via the Internet at http://pubs.acs.org

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Notes
The authors declare no competing financial interest.
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REFERENCES


(3) Han, J.; Burgess, K. Chem. Rev. 2010, 110, 2709.


