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Water-soluble pyrrolopyrrole cyanine (PPCy) near-infrared fluorescent pH indicators for strong acidity†

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Water-soluble, fluorescence-switchable derivatives of pyrrolopyrrole cyanines (PPCys) which serve as near-infrared pH indicators have been synthesized. The dyes reported have pK_a values of 2.4–3.4 and thus are suitable for sensing in highly acidic environments. The new pH probes were evaluated by fluorescence imaging experiments in a cellular environment.

Fluorescence based sensing has become an important technique in biomedicine. A number of these methods, such as ophthalmological angiography based on indocyanine green fluorescence[1] or intrasurgical tumor targeting based on administration of 5-aminolevulinic acid[2] are now routinely used. In general, for all medical applications deep penetration depths of the techniques are important. Therefore, due to the reduced scattering and low absorption at long wavelengths, the application of near-infrared (NIR) fluorophores is desirable.[3] Monitoring pH is crucial for the understanding of physiological and pathological processes in organisms. Changes in pH values also play a pivotal role on cellular events, such as signal transduction, autophagy or apoptosis.[4] While many pH fluorescence indicators have been designed to work in the pH range of roughly 6 to 8 or in the weak acidic range from 4 to 6,[5] hardly any pH-sensitive fluorophores working in the strong acidic range below pH 4 are known.[6] Such chromophores would, however, be of great interest for *e.g.* imaging the gastrointestinal tract.[7]

In this contribution, we report the development of novel water-soluble NIR fluorophores which exhibit strong fluorescence in the highly acidic range. The dyes are based on the recently developed pyrrolopyrrole cyanines (PPCys) which themselves are derived from diketopyrrolopyrroles (DPPs).[8] PPCys are highly fluorescent and photostable NIR dyes which allow an easy tailoring of their absorption and emission properties by appropriate substitution patterns.[9] Their structure–property relationships meanwhile are well understood. This allowed *e.g.* the development of PPCy dyes with an acid/base-dependent fluorescence switching by introducing aminophenyl donor groups into the PPCy skeleton.[10] Those derivatives, as nearly all PPCy dyes reported to date, however, are only soluble in nonpolar solvents. Only very recently, the synthesis of the first water-soluble PPCy dyes by a postsynthetic modification route was reported,[11] which yielded ionic (tetrasulfonated) PPCys with high water-solubility (mM range). This opens way for integrating the excellent photophysical properties of PPCys as NIR dyes into water-soluble pH indicators. Our first attempt into this direction was the development of such dyes by transferring the established synthetic route for water-solubilisation onto the described fluorescence-switchable derivatives. Yet, this strategy failed (for synthesis scheme of the failed route see Fig. S1 in ESI†). We have engineered a new postmodification route which yields non-ionic PPCys with high water-solubility. This synthetic strategy is applicable to the aminophenyl substituted, fluorescence-switchable PPCys. We describe the synthesis and spectroscopic characterization of four PPCy based near-infrared pH indicators. These dyes exhibit pK_a values between 2.4 and 3.4 and thus are applicable in strongly acidic environments. Fluorescence imaging demonstrates their great potential as pH indicators on a cellular level. As other PPCys,[12] we envision that the new dyes can also be employed for live animal imaging.

The synthetic route (Scheme 1; for Experimental section see ESI†) to the novel PPCy pH indicators follows the introduction of terminal alkynes into the dye structure, as has partially been described earlier by Zhou *et al.*[13] For this purpose, the dyes are postsynthetically modified by the Cu(I) catalyzed alkyne azide cycloaddition (CuAAC) with poly(ethyleneglycol)azide (PEG-N_3). Aminophenyl substituted DPP **1** is reacted with two equivalents of 2-(6-((pent-4-yn-1-yloxy)methyl)quinolin-2-yl)acetonitrile **2′** or

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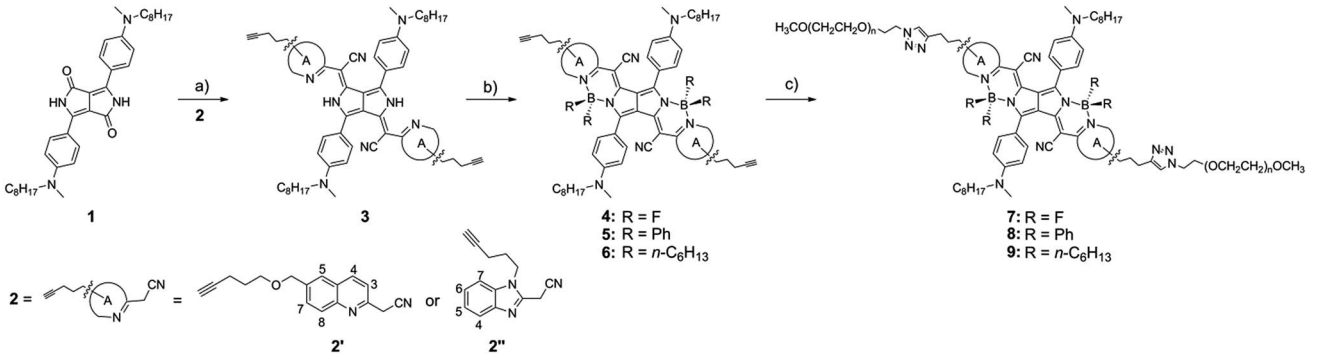
† Electronic supplementary information (ESI) available: Synthesis and characterization of intermediates and final compounds. Synthesis scheme of failed route to sulfonated, aminophenyl substituted PPCys. Fluorescence titration spectra of **7′**, **9′** and **8″**. Absorption spectra of final compounds **7′**, **8′**, **9′** and **8″** in CH_2Cl_2 and H_2O. See DOI: 10.1039/c5ra04812e



Scheme 1 *Reagents and conditions* (A = aromatic heterocycle): (a) anhydrous toluene/$POCl_3$, reflux; (b) CH_2Cl_2/diisopropylethylamine and either $BF_3{\cdot}OEt_2$ (**4**) or BPh_2Cl (**5**) or $BHex_2Cl$ (**6**), reflux; (c) degassed DMF/$CuSO_4{\cdot}5H_2O$/Na-L-(+)-ascorbat/PEG-N_3, room temperature.

2-(1-(pent-4-yn-1-yl)-1*H*-benzo[*d*]imidazol-2-yl)acetonitrile **2″**, respectively, by activation through phosphoryl chloride to yield the hydrogen chelates **3′** or **3″**, respectively. These H-PPCys **3** are converted to the quinoline substituted BF_2-PPCy **4′**, BPh_2-PPCy **5′**, $BHex_2$-PPCy **6′** or to the benzimidazole substituted BPh_2-PPCy **5″**, respectively. In the case of benzimidazole, only the BPh_2-PPCy **5″** was synthesized. These four BR_2-PPCy dyes were then reacted with PEG-N_3 to yield the water-soluble, quinoline substituted PEG-BF_2-PPCy **7′**, PEG-BPh_2-PPCy **8′**, PEG-$BHex_2$-PPCy **9′** and the benzimidazole substituted PEG-BPh_2-PPCy **8″**, respectively.

The obtained PEG-BR_2-PPCys show a high solubility not only in water, but also in organic solvents like dichloromethane, methanol or DMSO, owing to the nature of the long PEG chains. The reactant PEG-N_3 was commercially available (average $M_n = 1000$ g mol^{-1}, PDI < 1.1, Sigma-Adrich) and, according to MALDI mass spectra of the final compounds, PEG-chains with ethyleneglycol repeating units of an average n = 22–24 were incorporated. The pK_a values of the amino functions at the DPP core were determined *via* fluorescence titration experiments, using the *Henderson–Hasselbalch* equation,

$$\mathrm{pH} = \mathrm{p}K_a + \log \frac{c(\text{PPCy-NR}_2)}{c(\text{PPCy-NR}_2\text{H}^+)}$$

where c(PPCy-NR_2) is the concentration of dye with free and c(PPCy-NR_2H^+) the concentration of the dye with protonated amino group(s). For c(PPCy-NR_2) = c(PPCy-NR_2H^+), which is true for the fluorescence intensity at half its maximum, the pK_a equals the pH value. As it was seen in earlier protonation experiments of aminophenyl substituted PPCys, the two amino functions are successively protonated.[10] However, the second protonation occurs very rapidly after the first one, so that the two very slightly different pK_a values may for practical purposes be regarded as one. Fig. 1 shows the pH dependent fluorescence spectra of quinoline substituted PEG-BPh_2-PPCy **8′** in water : methanol (1 : 1) by protonation through HCl. At λ^F_{max} = 820 nm the ratio between the fluorescence intensity at a certain pH and the maximum intensity was plotted against the pH and a sigmoidal fit revealed the pK_a value of 3.2, meaning PPCy **8′** turns on strong emission at low pH values (inset in Fig. 1; for fluorescence titration spectra of **7′**, **9′** and **8″** see Fig. S2–S4 in ESI†). The quinoline substituted PEG-BF_2-PPCy **7′** and PEG-$BHex_2$-PPCy **9′** were synthesized to clarify the different electronic effects of the complexing groups on the pK_a values of the amino functions. The likewise determined pK_a values of these derivatives were 2.4 and 2.5 (Table 1), respectively, demonstrating that the complexing group has only a weak effect on the pK_a value. Regarding these results, we were interested in synthesizing a dye with different heteroaromatic end groups to determine the influence of these groups on the pK_a value. Therefore, benzimidazole substituted PEG-BPh_2-PPCy **8″** was synthesized and its pK_a value was determined to 3.4. This result shows that the pK_a depends only weakly on the substitution pattern of the PPCy and instead is mainly determined by the aminophenyl structural motif.

An important characteristic of a pH fluorescence indicator especially for imaging applications is a good contrast in fluorescence quantum yields Φ_F between the fluorescent and the

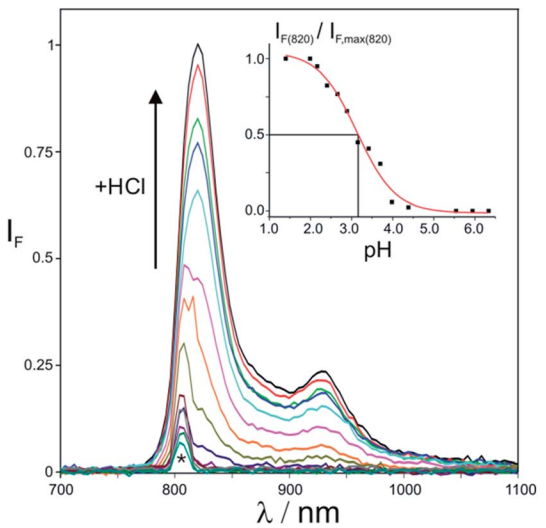


Fig. 1 Fluorescence spectra of PEG-BPh_2-PPCy **8′** in water : methanol (1 : 1) by titration with HCl (asterisk: stray light of λ_{exc} = 804 nm); inset: determination of the pK_a value by plotting the ratio between the fluorescence intensity at a certain pH and the maximum intensity against the pH and sigmoidal fitting.

Table 1 Spectroscopic data of unprotonated and protonated (in brackets) PEG-BR_2-PPCys in dichloromethane and water. λ^{A}_{max}: wavelength of the absorption maximum, λ^{F}_{max}: wavelength of the fluorescence maximum, Φ_F: fluorescence quantum yield

	Solvent[a]	λ^{A}_{max}/nm	λ^{F}_{max}/nm	Φ_F	pK_a[b]
7′	DCM	800 (753)	— (763)	0 (0.53)	2.4
	H_2O	809 (676)[c]	— (750)	0 (0.01)	
8′	DCM	831 (822)	850 (830)	0.08 (0.52)	3.2
	H_2O	818 (806)	— (825)	0 (0.03)	
9′	DCM	839 (843)	857 (855)	0.24 (0.25)	2.5
	H_2O	828 (834)	— (—)	0 (0)	
8″	DCM	756 (757)	823 (781)	0.03 (0.29)	3.4
	H_2O	761 (760)	— (793)	0 (0.02)	

[a] Trifluoroacetic acid (TFA) and conc. HCl were used for protonation in dichloromethane and water, respectively. [b] pK_a values were determined in H_2O : MeOH, 1 : 1. [c] H-aggregation band (monomer band at 739 nm).

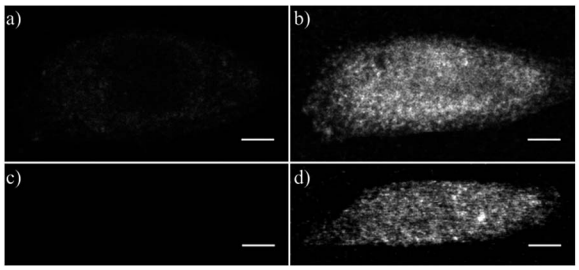


Fig. 2 Confocal fluorescence images of fixed CHO cells after 30 minutes of incubation with a 1 μM solution of novel PPCy pH indicators: PEG-BPh_2-PPCy 8′ at (a) neutral pH and (b) pH = 1; PEG-BF_2-PPCy 7′ at (c) neutral pH and (d) pH = 1. For comparison, grey scales of images (a) and (c) were adjusted to those of (b) and (d), respectively (excitation wavelength: 690 nm, detection wavelengths: (a and b) >770 nm; (c and d) >710 nm, see ESI†); scalebars: 5 μm.

"non-fluorescent" form. Ideally, the fluorescent form (in this case the protonated PPCy) has a high and the "non-fluorescent" form (unprotonated PPCy) has a quantum yield of nearly zero. It was found that unprotonated PEG-BPh_2-PPCy 8′ has a $\Phi_F = 0$ in pure water (the same as for 7′, 9′ and 8″; *cf.* Table 1), but it was shown in previous work that in biological (cellular) environments, the photophysical properties of PPCys are not corresponding to a purely aqueous surrounding.[11] To examine whether unprotonated 8′ also shows no fluorescence in a biological environment, fluorescence images were taken on fixed CHO cells incubated with a 1 μM solution of 8′ for 30 minutes using a scanning stage confocal fluorescence microscope. Fig. 2a indeed shows a weak emission of 8′ in the cytoplasm by excitation with a 690 nm laser, revealing a solvation effect of the PPCy which is different from that of a purely aqueous surrounding. After permeabilization of the cells and adjusting the external cellular medium to pH = 1, PPCy 8′ shows the expected intense fluorescence (Fig. 2b). This result documents a diminished contrast between the two forms of the dye in the cellular environment. Moreover, it is thereby shown that non-ionic (PEGylated) PPCys enter the cells without application of any permeability-enhancing techniques.[11] The same experiment was performed with PEG-BF_2-PPCy 7′. Fig. 2c and d show the fluorescence images at neutral pH and pH = 1, respectively. While at low pH there is a similar strong emission as in the case of 8′, no fluorescence in the unprotonated state is observed at all, meaning an optimal contrast between the two forms of PPCy 7′. In fact, it was not possible to locate cells by fluorescence imaging at neutral pH, so the pH was first adjusted to 1 (Fig. 2d) and then readjusted to neutral again (Fig. 2c). This also demonstrates that protonation of aminophenyl substituted PPCys in CHO cells is reversible.

An explanation for the better contrast in the case of PEG-BF_2-PPCy 7′ compared to PEG-BPh_2-PPCy 8′ can be found in the absorption spectra of these compounds (see Fig. S5 and S6 in ESI† for absorption spectra of 7′, 8′, 9′ and 8″ in CH_2Cl_2 and H_2O, respectively). As has been explained in an earlier publication,[10] the intensity of the charge transfer (CT) transition band is a measure of the coupling strength of the aminophenyl donor substituent to the chromophoric system and hence an indicator for the magnitude of the fluorescence quenching in the unprotonated form. The intensity of the CT band in the unprotonated form is much higher for PEG-BF_2-PPCy 7′ than for PEG-BPh_2-PPCy 8′, resulting in a fluorescence quantum yield of 0 compared to 8% in dichloromethane, respectively (*cf.* Table 1). Comparing the quantum yields in an organic solvent is sensible since, as described above, the solvation effect observed in cells is different from the pure aqueous milieu (Fig. 2a). For PEG-$BHex_2$-PPCy 9′ the CT band intensity is even lower than in the case of 8′, which means that the coupling of the aminophenyl donor to the chromophore is very weak. This results in practically no contrast of the quantum yields between the unprotonated and protonated form of 24% to 25%, respectively. Benzimidazol substituted PPCy 8″ again shows a better contrast in quantum yields of 3% to 29%, as its CT band intensity is higher.

In conclusion, we report the synthesis of four novel pyrrolopyrrole cyanine dyes with high water-solubility which act as near-infrared fluorescent pH indicators. The synthesis follows a postmodification of PPCys with terminal alkynes in a Cu(I) catalyzed alkyne azide cycloaddition (CuAAC) with poly(ethyleneglycol)azide. The pK_a values of the four PPCys are 2.4, 2.5, 3.2 and 3.4 (water : methanol, 1 : 1), respectively, thus having a highly acidic working range. PEG-BF_2-PPCy 7′ and PEG-BPh_2-PPCy 8′ have been tested as pH indicators in CHO cells and it was found that 7′ shows an optimal contrast between the non-fluorescent (unprotonated) and the fluorescent (protonated) form, excelling this derivative as an ideal near-infrared pH indicator for strong acidity.

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