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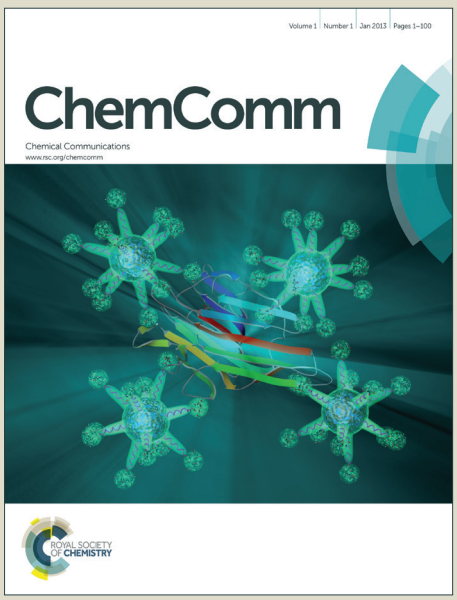

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FRET based pH probe with broad working range applicable to referenced ratiometric dual wavelength and luminescence lifetime read out†

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A luminescent probe for determination of pH was designed based on a Förster resonance energy transfer (FRET) system, combining a europium chelate as donor and carboxynaphtho-fluorescein as pH sensitive acceptor. The FRET system enables referenced pH detection in an exceptional broad dynamic range from pH 3 to 9.

Up to now, there is still an unfulfilled demand for cheap, simple, and reversible optical chemical sensors for the determination of ubiquitous analytes such as pH, oxygen or certain metal ions. Particularly, the utilization of photoluminescent probes as signal transducer exhibits some major advances compared to sensors based on electrodes. They are not interfered by electrical or magnetical fields, and sensor elements can be operated in a more or less noninvasive and remote way and miniaturized to the nanoscale level. Luminescent probes for determination of pH have been synthesized in a high versatility for different kind of applications with a focus on indicators for intracellular measurements and imaging in recent years[1]. Likewise, optical sensors for pH determination can be designed and fabricated in a high variety, including fiber optic sensors[2], sensor spots integrated in reaction vessels or microwell plates[3], 2-D sensor layers for medical imaging[4] or marine research[5], as well as nanoprobes for intracellular and in vivo measurements[6]. However, luminescent sensors always require referencing of the responsive signals because measurements based only on luminescence intensity are not reliable and prone to interferences. Two methods to reference the pH response of luminescent indicators are established[7]. One is the use of dual wavelength (2-λ) probes such as HPTS[8] or certain carboxy-fluorescein or naphthofluorescein derivates[9]. This is straightforward and the underlying principle is that the acidic (protonated) and basic (deprotonated) forms of the dyes have different excitation and/or emission maxima. This can be used for intrinsically referenced ratiometric measurements by recording the sensor response at two different excitation or emission wavelengths. An alternatives is the addition of a reference dye[10], which is inert to pH and can be excited with the same light source.

The second important referencing method is the application of time-resolved luminescence techniques such as fluorescence lifetime imaging (FLIM)[11], and the time-domain[12] or frequency domain[13] lifetime referencing. Besides the issue of referencing, background luminescence and light scatter is a major problem for optical sensing and imaging of pH, particularly in biological, highly colored or dispersive samples. This concerns primarily fluorescent indicators with shortwave excitation and emission and small Stokes shifts. On the other hand, NIR dyes that enable excitation in the biological optical window are commonly outmost prone to photobleaching, although there is some progress in the development of stable NIR probes for pH.[14] The combination of dyes to FRET-based donor-acceptor systems represents another valuable strategy to achieve ratiometric pH indicators. For example, the conjugation of coumarin and rhodamin leads to a dual excitation wavelength probe,[15] the conjugation of coumarin and rhodamine B to a dual emission wavelength probe.[16] FRET systems for intracellular measurements were assembled from quantum dots and a pH-sensitive fluorescent protein[17] or from two fluorescent proteins (YFP and ECFP).[18]

Hence, there is still a demand for luminescent probes that are appropriate for background-free and intrinsic referenced pH sensing using straightforward detection techniques. One option is the utilization of lanthanide luminescence. Sensitized europium (or terbium) chelates provide large shifts between the excitation wavelength of the sensitizing chromophores (usually in the range from 320 to 400 nm) and the bright main emission line of europium in the red spectral range (~ 615 nm). Thus, the luminescence emission can be easily separated from the excitation light by optical filters. Furthermore, the emission of europium occurs with a slow decay, providing luminescence lifetimes of > 100 µs. These can be recorded and imaged with a simple and inexpensive instrumentation. Time-resolved (gated) luminescence detection enables the elimination of autofluorescence, which decays within nanoseconds, and provides an intrinsically referenced signal which does not depend on the concentration of the dye.

One strategy to utilize lanthanide luminescence for pH sensing is to apply sensitizing chromophores that respond to pH such as phenanthridine. These can be attached to chelating ligands for europium or terbium derived from DOTA (1,4,7,10-tetraaza-cyclo-dodecane-1,4,7,10-tetraacetic acid.[19] Protonation of the sensitizer affects the energy transfer to the europium and therefore its luminescence intensity and lifetime. Related complexes were designed for intracellular pH measurements.[20] The strategy of using Förster resonance energy transfer (FRET) systems for pH sensing

including a lanthanide complex as donor and a pH sensitive dye as acceptor was drafted by *Lakowicz et al*.[21], but no experimental data have been shown. Later, *Kessler* prepared a FRET complex between a sensitized europium terpyridine complex and the non-fluorescent colorimetric pH indicator bromothymol blue (BTB) in aqueous solution for pH sensing and studied the involved processes.[22] A luminescence lifetime based pH sensor was fabricated by the immobilization of a sensitized europium chelate based on a diethylenetriamine pentaacetic acid ligand together with BTB in a sol-gel membrane.[23] This ligand system was modified with a hydrazid group to achieve a europium complex which luminescence intensity and lifetime directly responds to pH.[24]

However, lanthanide probes based on the protonation of the sensitizing chromophore provide only confined changes of luminescence intensity and lifetime resulting in limited resolution and dynamic ranges. On the other hand, BTB is a dark quencher and does not enable 2-λ read out. Moreover, it forms indicator systems with the europium donor that cover a dynamic range of maximal 3 pH units, which applies to most pH-sensitive probes. "Broadband" pH indicators on the basis of asymmetric perylene bisimides have been reported with a dynamic range of over 4 pH units[25].

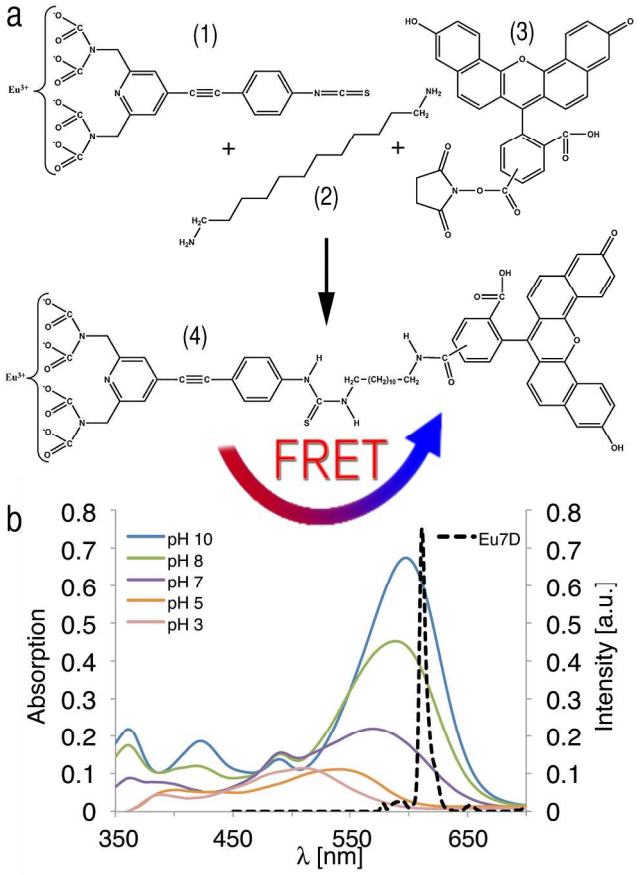

Figure 1. **(a)** Synthesis of the molecular FRET system consisting of a sensitized europium chelate Eu7D (**1**) containing an isothiocyanate function and CNF (**3**) linked by 1,12-aminododecane (**2**). **(b)** Red luminescence emission of the europium chelate (dashed line) and absorption of CNF at different pH (from 10 to 3, colored lines)

Therefore, we designed a molecular probe (Figure 1a) for pH sensing which originates from a completely different strategy. It is composed of a sensitized europium complex Eu7D (**1**) that is coupled via a dodecyl spacer (**2**) to the fluorescent NIR emitting pH indicator 5(6)-carboxynaphthofluorescein (CNF) (**3**). The C_{12} spacer enables a highly efficient pH-dependent FRET from the emitting state of the europium center (donor) to CNF (acceptor) which has a broad pH-dependent absorption between 550 and 650 nm (Figure 2b) with a pK_a of 7.6. This absorption diminishes with decreasing pH, leading to a less strong quenching of the europium luminescence at 615 nm (Figure S1in the Supplementary Information). An inherent difference of this system compared to the FRET based indicators described above is that it enables time-resolved FRET (TR-FRET) measurements.

Therefore, the resulting pH sensitive probe (**4**) permits the application of two different intrinsically referenced detection methods, either in the 2-λ mode recording the red emission of europium at 615 nm and the FRET-sensitized NIR emission of CNF at 670 nm, or the measurement of the change of the luminescence lifetime of the red europium emission (time-resolved FRET mode, TR-FRET) .

This FRET system has some unique properties which makes it outstanding from other probes for pH. First, the seven-dentate europium chelate Eu7D provides stable luminescence intensities and lifetimes of the 615 nm peak free from common interferences[26] and is stable in acidic environment until a pH of 3. At lower pH, decomposition takes place (Figure S2). The 615 nm emission has a luminescence lifetime τ around 380 µs in aqueous solution at pH 7.[27] Its quantum yield of 0.06 is comparatively high for europium complexes in aqueous solution.[28] This lifetime is reduced to around 70 µs after conjugation to CNF. According to equation 1, a FRET efficiency η of 0.82 can be calculated at pH 7. At pH 8 the FRET efficiency amounts already 0.97.

$$\eta_{FRET} = 1 - \frac{\tau(\text{Eu-CNF})}{\tau(\text{Eu})} \qquad (1)$$

with τ(Eu) as the luminescence lifetime of the Eu emission and τ(Eu-CNF) as the lifetime of the Eu-CNF conjugate.

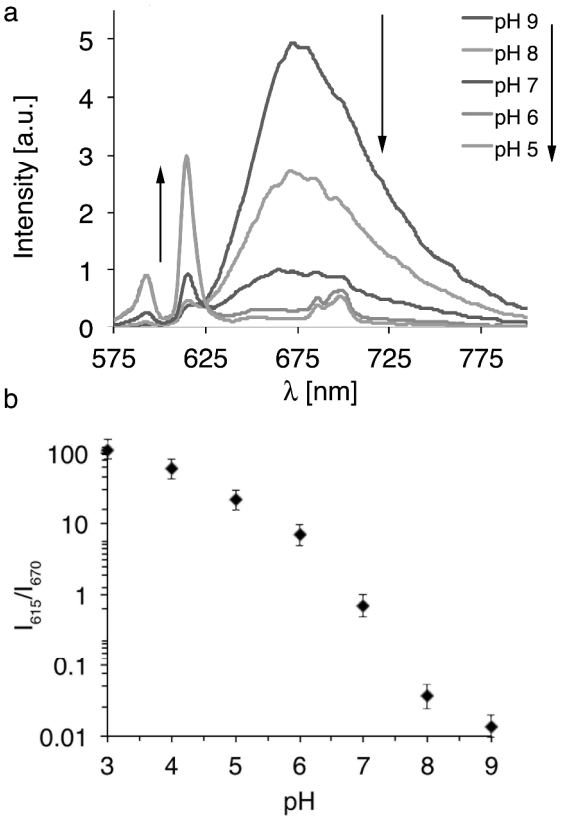

Figure 2. **(a)** Luminescence spectra of the FRET system (**4**) at different pH from 9 to 5. **(b)** Ratiometric luminescence response calculated from the peak ratio 615 to 670 nm displayed as semilogarithmic plot and standard deviations (5-10 % in the range between pH 3 and 7; N=3).

This very effective energy transfer makes this donor/acceptor system highly sensitive for protonation of the acceptor, because the pH dependent changes of the absorption of CNF generate a distinct signal response of the sharp line-like red emission of the europium ion because the spectral overlap (J) of the FRET pair is modulated by the pH.

The 2-λ read out of the probe is shown in Figure 2. The FRET coupling of the two luminophores causes a strong increase of the europium emission and parallel decrease of the FRET-sensitized emission of CNF at 670 nm (Figure 2a) in acidic environment. The small peak around 690 nm is the second order peak of the excitation light (345 nm monochromator) and does not affect the intensity at 670 nm. Moreover, it can be eliminated using a spectrometer with a time gated read out mode as applied in Figure S3.

Figure S3 shows the signal enhancement of the europium emission with decreasing pH in more detail. These opposing trends lead to a powerful signal response in case of a ratiometric evaluation. Thereby, the color of the probe changes from deep blue at pH 9 to light purple at pH 3 (see Figure S4). Figure 2b shows the calculated signal ratios recorded at 615 nm and 670 nm in a semi-logarithmic plot. After division (I_{615}/I_{670}), the calibration plot covers a range from 0.01 and 100. This represents a remarkably large ratiometric signal response of over four orders of magnitude. This unique feature of the probe enables a referenced pH sensing with high resolution (low limit of detection). Furthermore, determinations in a very broad dynamic range from pH 3 to pH 9 are achievable. Thus, six pH units are covered, which cannot be achieved with other luminescent pH indicators so far reported in literature. The response occurs fast (t_{90} < 1 s) and completely reversible (see Figure S5).

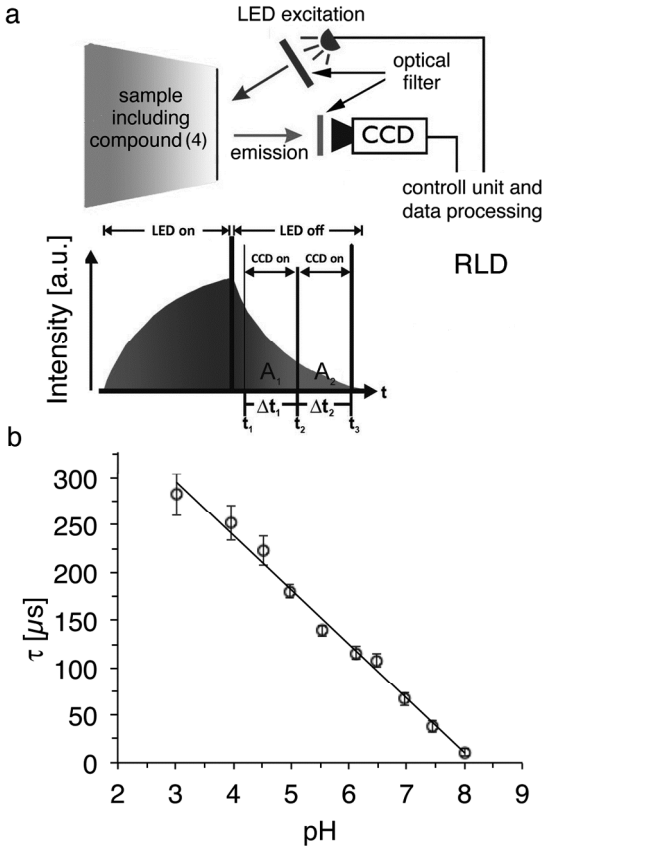

Figure 3. (a) Scheme of the instrumental set up and measurement principle (RLD) of the time-gated luminescence imaging method applied for the determination of the luminescence lifetime changes of (**4**) to pH. For details of the parameter settings see the Supplementary Information. **(b)** pH dependent luminescence lifetimes including error bars (N=3) with linear fit.

Time-gated luminescence detection represents the second option for referenced measurements. For this purpose, we use an imaging system shown in Figure 3a suitable for time-resolved fluorometry. The data acquisition, integrating the luminescence intensity in two subsequent time gates after a short excitation pulse, is carried out on the basis of the rapid lifetime determination (RLD) method.[29] The imaging set up and the measurement principle have been previously described in detail.[30] The intensity ratio A_1/A_2 depends only on the change of the luminescence decay, but not on the intensity and therefore on the concentration of the probe.

Assuming a monoexponential decay, the corresponding lifetimes τ can be calculated according to equation 2.

$$\tau = \frac{t_2 - t_1}{\ln A_1 / A_2} \qquad (2)$$

Figure 3b shows the resulting calibration plot. The dynamic range of the time-gated detection method covers pH values from 8 to 3. Thereby, the lifetimes increase from 25 to 280 µs. It has to be noted that the decay is not monoexponential, thus the lifetime values calculated here represent only a rough average quantity. However, for sensing applications the relative changes of the lifetime is the relevant quantity and these can be determined with the RLD method quite accurately. Alternatively, the intensity ratios A_1/A_2 can be plotted instead of the lifetimes. Decay curves are shown in Figure S6. In this pH range, the unmodified europium chelate shows constant lifetimes (Figure S7). The europium luminescence gets too weak at higher pH to enable reasonable lifetime measurements. Of course, this range can be extended by using higher concentrations of the probe. Standard deviations can be calculated either on the basis of repetitive series of measurements (e.g. for n =3 in Figure 3b) or on the basis of the pixel to pixel variations in a defined region of interest (ROI) of the lifetime image (spatial standard deviations, see Figure S7). In both cases the physiological important pH range from 8 to 5 shows a rather linear response with spatial standard deviations of maximal 10%. This yields a resolution (limit of detection) for pH sensing of ± 0.1 pH units.

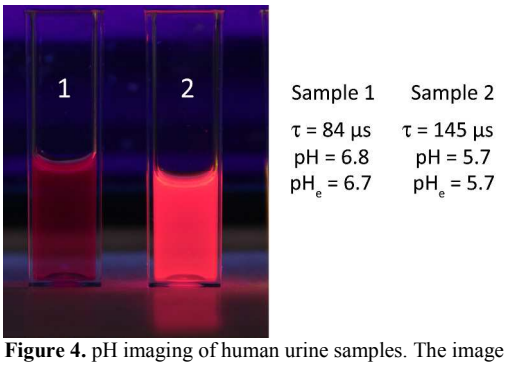

Figure 4. pH imaging of human urine samples. The image depicts real color photographs of urine samples with added luminescent pH indicator (c = 100 µmol L^{-1}) under UV light. Sample 1 is the reference containing urine of a test person. Sample 2 was taken 12 hours subsequent to the intake of vinegar (5 mg diluted in water). Lifetime images were acquired with the time-resolved imaging set up and pH values calculated from the linear calibration. pH_e = pH values obtained from reference measurements with a pH glass electrode.

We tested the applicability of the probe in biological fluids by means of human urine samples. Figure 4 shows the results from two different samples, one taken before and one 12 h after ingestion of 5 mg of vinegar. The luminescence lifetimes of the pH indicator were

determined by the RLD method using the imaging set up and the corresponding pH values were calculated from the calibration curve. The found pH values are in good accordance with the pH values determined with a standard pH glass electrode as control (for more details see the Supplementary Information).

In conclusion, we have shown that the coupling of a europium chelate to the pH indicator CNF forms a FRET system that responds to pH with high sensitivity due to a pH dependent change of its spectral overlap. This broadband pH probe covers a dynamic range from pH 9 to pH 3 and permits referenced measurements either by ratiometric 2-λ or by time-resolved (time gated) detection methods. Due to background luminescence reduction, the latter can be applied to turbid, colored or highly scattering biological samples, such as urine. The measurement range is more than sufficient to monitor pH changes in the physiological range of living systems. The probe is photostable and reacts fast and reversible. As in case of every pH indicator the response of CNF is affected by ionic strength. This has to be considered at the calibration of the indicator. Further studies will be carried out to explore whether this TR-FRET probe can be integrated in optical sensor films or be used for intracellular pH sensing and imaging, for example by combination of Eu7D with the cell-permeable diacetate of CNF.

Notes and references

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† Electronic Supplementary Information (ESI) available [including experimental part (materials and methods). chemical synthesis and characterization, additional spectral data, experimental details]. See DOI: 10.1039/c000000x/

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