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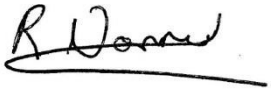

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Journal Name

COMMUNICATION

Silica supported tricarbocyanine based pH nanosensor with large Stokes shift and near infrared fluorescent response: performance in vitro and live cells

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We report the synthesis of a near-infrared (NIR) fluorescent pH probe with a remarkable Stokes shift reported (~ 135 nm) based on a tricarbocyanine (Cy-PIP). The fluorescent molecule was anchored to SiO_2 nanoparticles (Cy-PIP@SiO_2) and is capable of monitoring pH changes within the physiological range (pH 6-8). The Cy-PIP@SiO_2 nanoparticles were succesfully internalized by HeLa cells as shown by fluorescence confocal microscopy, while flow cytometry revealed pH fluctuations during the endocytic pathway.

Extra- and intracellular pH measurements are of fundamental importance when understanding physiological/biological processes associated with environmental acidic changes: endocytosis, metabolism, ion transport or cancer.[1] For instance, altered metabolism in tumour cells lacking oxygen and nutrients leads to high levels of lactic acid, affecting drug permeability or promoting tumour spreading.[2, 3] In this context, luminescent transduction and sensing of biological events fuels the research on molecular and nanosized sensor probe systems with reliable and sensitive signals, from single organic fluorophores[4] to integrated macro/nanosized emitters. [5, 6] In addition, non-invasive techniques for monitoring biodistribution of nanomedicines based on optical imaging are greatly attractive due to their ease of use and simple synthesis. Clearly these new techniques will have to focus on the optical properties of human subcutaneous tissues as they determine a "biological window" in the near-infrared region (NIR) of the spectrum (650-900 nm). The synthesis of fluorophore molecular probes and lanthanide-based upconversion nanocrystals with NIR activity is of biological and medical relevance.[7-11] These systems take advantage of the deep tissue penetration of this wavelength range and the minimal background signal from intrinsic cellular components. Evidently, these features stand out when compared with positron emission tomography (PET) and single photon emission computed tomography (SPECT) techniques which require handling of radiolabeled compounds (with limited decay half-life) and highly engineered detectors setup.

Most of the available fluorophores that are pH-sensitive in the physiological range operate within the visible region with small Stokes shifts and have the additional complication of limited water solubility requiring the use of cytotoxic co-solvents.[4, 12, 13] Given the scarce offer of truly NIR small fluorophores, tricarbocyanines emerge as very convenient molecules in terms of synthetic versatility, water solubility and optical properties.[14] Moreover, a high rated property of these probes among other cyanines is the large Stokes shift between excitation and emission (>100 nm) when an amine group is included in the *meso* position; this minimizes self-quenching and the influence of light scattering.[15] Our group has previously shown the excellent performance of this type of NIR-dyes as probes or sensors in living cells.[16-19] In particular, pH probes derived from tricarbocyanines with piperazine, terpyridine moieties or the non-substituted nitrogen atom on the heterocyclic ring as receptors for protons, have a good sensitivity towards pH changes. [20-22] Unfortunately, these dyes are poorly soluble in water or have pKa values out of the physiological pH range (pH ~ 6-8).

Following the strategy of immobilizing the dyes within or on the surface of an inert matrix, silica nanoparticles reveal as an excellent support material due to their stability, low toxicity and cost production for highly biocompatible labeling and

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Electronic Supplementary Information (ESI) available: Reagents and instruments, synthetic and labelling procedures, additional spectroscopic data, ^{1}H, ^{13}C NMR, HRMS, flow cytometry. See DOI: 10.1039/x0xx00000x

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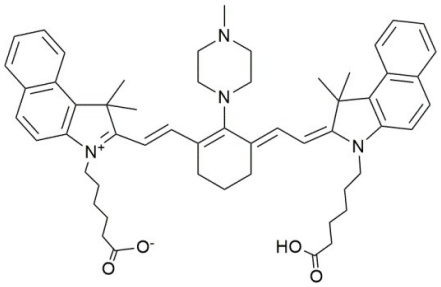
imaging agents that display enhanced fluorescence quantum yields and photostability.[23-25]

In this work, we combined the convenient optical properties of tricarbocyanines NIR-dyes with the versatility of silica based materials obtaining a large Stokes shift pH nanosensor. The NIR tricarbocyanine dye with a piperazine moiety acts as the proton acceptor and modulates the fluorescence. Moreover, we describe the preparation, characterization and sensing application of this novel pH-responsive NIR-nanosensor *in vitro* and in cultured HeLa live cells, monitoring pH changes by means of flow cytometry and confocal fluorescence microscopy after nanoparticle internalization, as proof of principle of the sensing performance in a biological context.

The pH sensitive NIR dye Cy-PIP (Scheme 1) was prepared by the well-known substitution of the chlorine atom by *N*-methyl piperazine as the pH sensitive unit (see Supporting Information, Fig. S1). The reaction proceeds in moderate yields under mild conditions while the Cl position is an excellent anchoring point for sensor design. [16-19] The carboxylic acid groups present in its chemical structure are essential for the subsequent covalent conjugation of the NIR dye on the amino derivatized surface of the silica nanoparticles; besides, they help to improve the water solubility.



Scheme 1. Molecular structure of pH-NIR responsive tricarbocyanine (Cy-PIP)

The spectral properties of 5 μM Cy-PIP were examined at pH 7.3 in 10 mM phosphate buffer solution (PB) showing an absorption maximum at 680 nm and a fluorescence emission peak around 820 nm (λ_{exc} = 685 nm) (see Fig. S10, Supplementary Information), yielding a Stokes shift of 135 nm, the largest observed for a probe of this type. Large Stokes shifts between absorption and emission are highly advantageous for fluorescence based (bio)imaging studies because they increase the sensitivity of the luminescent signal as they reduce self-quenching and inner filter effects. Moreover, having a large Stokes shift within a single organic fluorophore avoids the need to construct or synthesize multicomponent systems.[26, 27]

The Cy-PIP fluorescence emission at 820 nm noticeably changes when titrated in the 3.8-10.9 pH range as shown in Figure 1. The near-neutral pKa value for the probe in aqueous solution was computed by non-linear regression of the emission at 820 nm affording a value of 7.1, well suited for physiological medium. The same analysis was also applied to the pH-dependent absorption spectra, yielding a pKa value of 6.9 in accordance to the emission based value (data not shown). An unexpected photophysical behavior of our Cy-PIP probe is that fluorescence emission decreases when the aqueous medium becomes acidic; this contrasts to structurally similar reported tricarbocyanines-based pH probes.[28] In these dyes, the probes operate through a PET (photoinduced electron transfer) process typically with *off-on* response type moving from basic to acidic pH. [28] Since we are using a low dye concentration (~5 μM) for the fluorescence measurements, we can rule out aggregation of the dye as a source of luminescence quenching at acidic pH values. We speculate that other structural and electronic factors may be active along the titration complicating the photophysical analysis.

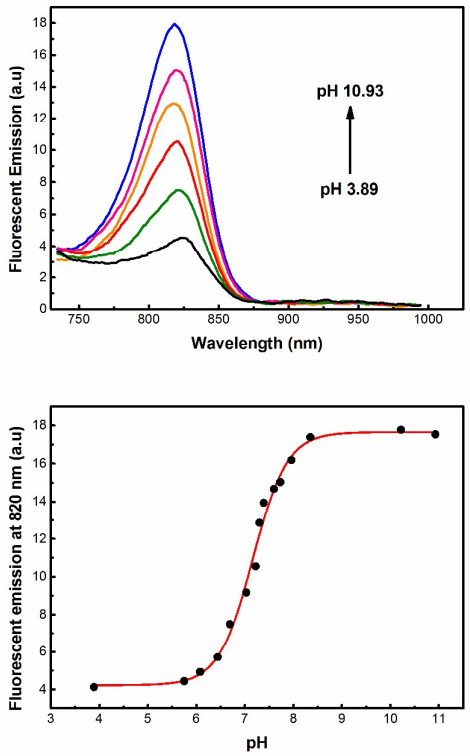


Figure 1. Emission spectra of Cy-PIP in 10 mM PB within the pH range 3.9-10.9 (upper panel); fluorescence intensity of Cy-PIP 5 μM at 820 nm within the pH range 3.9-10.9 (λ_{exc} = 680 nm, lower panel).

To test the photostability of the probe in aqueous solution, we measured the emission spectra in a time course of 2 hours at room temperature. The fluorescence emission does not change substantially in either acidic (pH=5.1) or basic (pH=9.4) buffer solutions (see Fig. S12, Supplementary Information), suggesting that the dye is relatively stable in aerated aqueous environments under continuous illumination.

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The surface of SiO_2 nanoparticles (NP) and colloids is an excellent interface for immobilization of sensor and actuator moieties.[23] In addition, organoalkoxysilane-based molecules constitute an ample and interesting chemical library for easy surface modification of siliceous and transition metal oxide materials.[29] With probe Cy-PIP in hand, amino derivatized silica nanoparticles were synthesized using 3-aminopropyltriethoxysilane according to standard reported procedures.[30, 31] The Cy-PIP pH-NIR sensor was anchored to the SiO_2 surface through an amidation reaction between the nanoparticles and the acidic groups of the probe (see Fig. S6, Supplementary Information). Typically, the centrifuged pellet acquired an intense blue color, in opposition to the pale white of the unmodified SiO_2 NP. This simple experimental observation was confirmed spectro-fluorometrically: the emission maximum of the anchored tricarbocyanine does not change significantly in comparison to the free dye and only a slight 5 nm bathochromic shift was observed in buffer solution at pH 7.3 (see Fig. S11, Supplementary Information). Moreover, SEM micrographs show uniform spherical particles with diameters less than 100 nm (see Fig S7, Supplementary Information) that are easily dispersed in aqueous solutions. Again, we observe a fluorescent emission that is pH dependent as in the NIR free dye case (see Figure 2, top and lower panel). When immobilized on the SiO_2 surface the NIR dye shows a pKa value of 7.2, obtained from non-linear regression analysis of the variation of fluorescence emission intensity at 820 nm with pH.[32] Typically, inorganic nano-particles smaller than 100 nm tend to show higher delivery efficiency for tumours. [33] Still, larger particles (~200 nm) can be also internalized by HeLa cells but at a slower rate. [34]

In order to evaluate the uptake and internalization of the nanoprobe, HeLa cells were treated for 1 hour with 2 μg/mL suspension of Cy-PIP@SiO_2 nanoparticles, washed with PB and incubated for different times at 37°C in culture media and fixed with 5% w/v paraformaldehyde.

Figure 3A shows that the Cy-PIP@SiO_2 NPs internalized after 1 hour incubation and progressively clustered within the perinuclear region as expected for the endocytosis process.[35] The cell viability was not affected during the experiment as a punctuated pattern could be observed with perinuclear localization after 180 min of incubation. This observation is consistent with cell binding and endosomal internalization of the probe.[36]

To assess quantitatively the pH fluctuation sensing performance of the probe in live cells, flow cytometry analysis was carried out in the same conditions as described for confocal microscopy. Typically, fluorescence intensity of $5 \cdot 10^4$ cells were analyzed for each incubation time and untreated cells were used as reference for fluorescence gating and histogram generation. In principle, mean fluorescence intensity showed a significant decrease over time. As shown in Figure 3B, after 240 minutes, the mean fluorescence signal decreased to 40 % whereas the high fluorescence population diminished 77.9 %. These observations are consistent with the mechanism where endosome-lysosome fusion leads to a drastic pH drop within the cellular compartment. [37, 38]

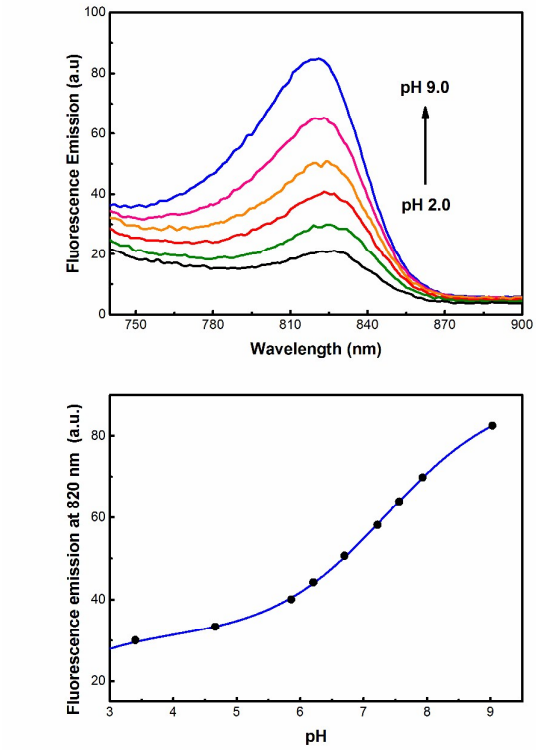

Fig. 2 Emission spectra of Cy-PIP@SiO_2 in 10 mM phosphate buffer solution within the pH range 2.0-9.0 (upper panel); changes of fluorescence intensity of Cy-PIP@SiO_2 at 820 nm within the pH range 3.0-9.0 (λ_{exc} = 680 nm, lower panel).

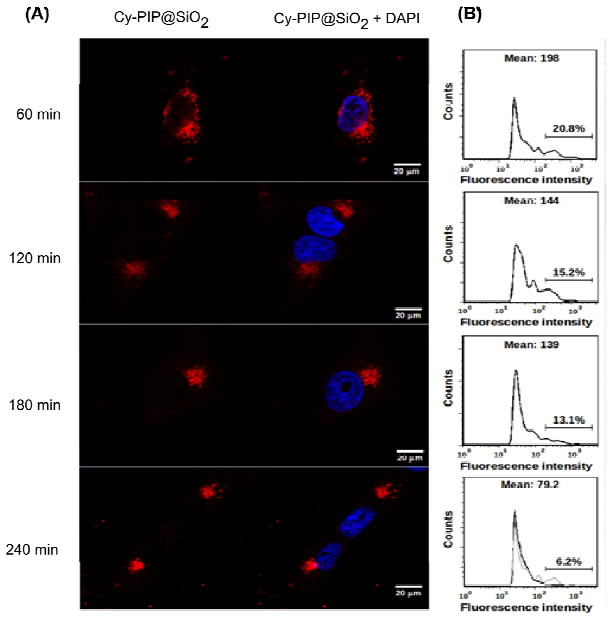

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Fig. 3 (A) Fluorescent confocal images of the Cy-PIP@SiO_2 pH NIR nanosensor in HeLa cells after incubation (cell nucleus were stained with DAPI). Laser excitation at 633 nm with filter APC-H7 and (B) flow cytometry measurements showing HeLa endocytic tracking.

Conclusions

Using affordable and versatile (in)organic synthetic methods we were able to construct a highly biocompatible nanosensor with pH monitoring capability. The exceptional features of this nanosensor are: a) the large Stokes shift of the NIR cyanine dye fluorescence emission, avoiding both cellular autofluorescence and photodamage while favoring deep tissue penetration in biological systems, as scattering and inner filter effects are minimized; b) matching of the *p*Ka NIR nanosensor with the physiological range of cellular *p*H with rapid and sensitive response to fluctuation and c) excellent performance in live cells *p*H monitoring with undetectable toxicity under the conditions tested. Clearly, this sensor platform establishes a promising starting point to achieve a high performance pH (bio)nanosensor. The flexibility of the SiO_2 surface and framework, in terms of chemical modification, offers a wide range of possibilities regarding the attachment of complementary probes and/or specific ligands to target and deliver the nanoprobe in a controlled manner. Moreover, the prospect to include a suitable fluorophore in the nanostructure to accomplish ratiometric response is currently under investigation.

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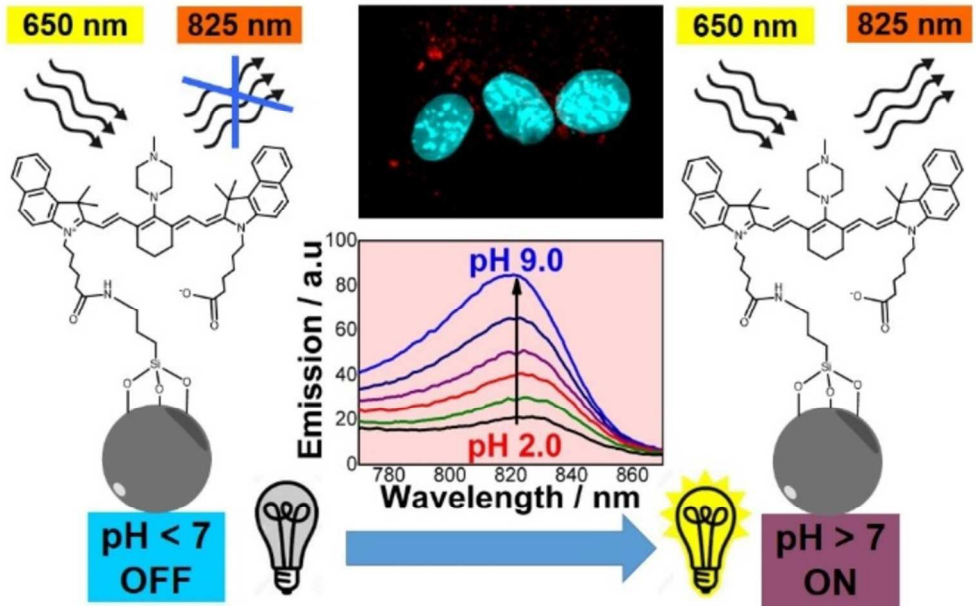

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