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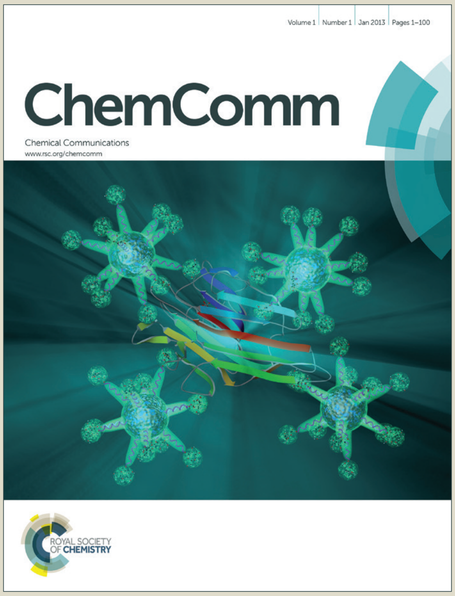

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Graphical abstract for

A Nanocellulose-Dye Conjugate for Multi-Format Optical pH-Sensing

by

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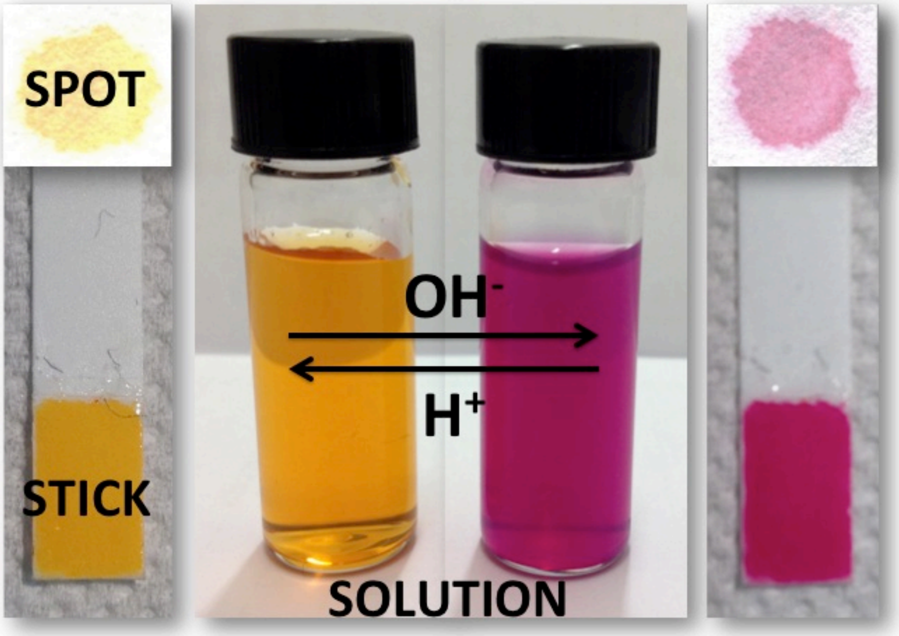

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A Nanocellulose-Dye Conjugate for Multi-Format Optical pH-Sensing

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A pH-sensitive azo-dye covalently grafted onto cellulose nanocrystals yields nanostructured optodes in the form of membrane, stick, and water-based ink for optical pH detection.

Thanks to its outstanding mechanical properties, cellulose has been chosen by Nature as a key structural component for trees, plants, algae, tunicates and bacteria.[1] In these biological structures, cellulose chains form highly hierarchical microfibrils that are held together by a dense network of intra- and intermolecular hydrogen bonds. Interestingly, microfibrils contain crystalline and amorphous cellulose domains that are randomly distributed along their length, a distinctive structural feature that opens up the top-down production of the so-called nanocrystalline cellulose (**NCC**).[2] In fact, a mild acid hydrolysis of various cellulosic sources promotes a preferential breakup of the microfibrillar amorphous regions producing a colloidal dispersion of cellulose crystallites having a characteristic rod-like morphology with lengths of 100-1000 nm and cross-sectional dimensions of 5-50 nm. By virtue of its structural and eco-sustainable features, **NCC** is now actively employed in a myriad of applications.[3]

A key strategy for tailoring the properties of **NCC** for specific applications is the effective grafting of functional molecules onto the surface of nanocrystals. We have recently reported a **NCC**-porphyrin hybrid that acts as a sensitizer for the production of singlet-oxygen under visible-light exposure.[4] Thielemans reported a nanocellulose densely decorated with ferrocenyl moieties that showed promising charge-transport properties.[5] The covalent attachment of luminescent tags to **NCC** including fluorescein,[6] methylcoumarin,[7] and dual labeling with fluorescein and rhodamine,[8] has been also successfully obtained.

Building on this concept, we show herein a straightforward synthetic method for the covalent attachment of a pH-sensitive dye to **NCC**, which exploits the vinylsulfone reactive moiety. Our synthetic design fulfills some of the most essential requirements for **NCC** functionalization, namely: 1) it capitalizes on the intrinsic reactivity of pristine **NCC**; 2) being a one-step procedure it minimizes reiteration of the mechanical operations (stirring, sonication, centrifugations, filtering, etc.) that could eventually degrade **NCC** morphology; 3) it takes place in aqueous milieu where **NCC** suspensions are less prone to aggregate and to settle down. The resulting material provides an innovative clue to nanostructured optodes, i.e. pH responsive nano-materials that are amenable to a multi-format application environment, with adaptive texture, form, and patterns. Our results address the fabrication of flat-membranes, pH-sensitive sticks, and printable inks, carved within the common motif of a biocompatible nano-cellulose matrix.

The **NCC** employed in this study was produced by hydrolysis of commercial microcrystalline cellulose from cotton linters (average size 20 μm, 80% degree of crystallinity) with 64% sulfuric acid for 45 minutes at 45°C. After usual work-up (*i.e.* centrifugation, extended dialysis against water, and desalting with ion-exchange resin) **NCC** was subjected to a size fractionation by ultracentrifugation discarding all the material that settled down after 1 hour at 9000 rpm. **NCC** was then recovered by ultracentrifugation at 12000 rpm for 5 hours.

The pH-sensitive dye **1** used for this study belongs to the class of Remazol dyes[9] which have been originally developed to react with hydroxyl groups of cotton fibers through a vinylsulfone reactive linker. Wolfbeis found an elegant way to exploit the same chemistry to produce chemical sensors supported onto cellulose acetate transparent membranes.[10a] Dye **1** was prepared as previously reported[10b] and covalently attached to **NCC** according to the reaction path depicted in Figure 1. Briefly, solid **1** was treated with the minimum amount of 96% sulfuric acid in order to convert its β-hydroxy-sulfonyl moiety into the corresponding [(sulfoxy)ethyl]-sulfonyl reactive group, **2**. Upon incresing the pH with 1M NaOH the solution turned from orange (neutral form) to purple (anionic form). pH was adjusted to 10 in order to promote the formation of the vinylsulfone reactive specie, **3**, then a suspension of **NCC** in water was added and stirred magnetically. After 2 hours the suspension was centrifuged at 12000 rpm to recover the purple **NCC**-dye adduct **5**. The solid was washed in water by several centrifugation/redispersion cycles then it was dialyzed for 6 days against water inside a dialysis membrane having a molecular-weight cutoff of 12 kDalton. The solid **4** was recovered by centrifugation at 12000 rpm and diluted to a concentration of 2.3% wt, as determined by weighting the residue left after evaporation at 80°C of a known amount of suspension.

There was some concern for **NCC** integrity since the strongly alkaline conditions required for the above reaction could be detrimental for nanocellulose, promoting both removal of sulfate

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esters and cellulose dissolution. In fact, recently MacLachlan described the use of **NCC** as a template for preparing mesoporous photonic resins in which the cellulose scaffold was removed by treatment in basic conditions.[11] However, atomic force microscopy (AFM) imaging of **4** (0.001% wt in water) showed persistence of the characteristic rod-like morphology of **NCC** (Figure 1a, close up of the marked region in Figure 1b). To assess the heights of the nanocrystals, 47 profiles of isolated objects were measured and a value of 4±1nm was obtained. Also, the widths and lengths of the nanocrystals were measured obtaining average values of 39±7 nm and 150±53 nm, respectively.

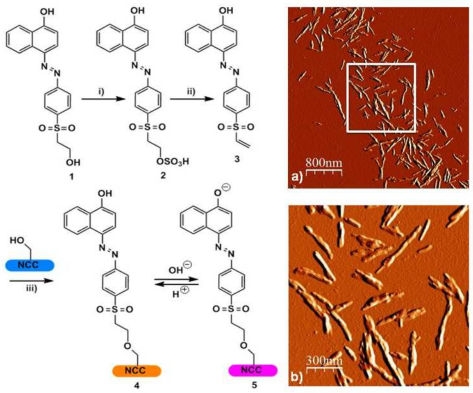

Figure 1: Synthesis of **4**: i) 96% H_2SO_4, 45 mins, 45°C, yield: 7.4%; ii) NaOH 1M until pH =10; iii) **NCC** suspension in water, yield: 37%. *Inset*: a) AFM image of **4**; b) AFM image of the marked region.

NCC-dye adduct **4** formed stable suspensions that gave the expected orange to purple colour change (Figure 2, bottom inset) upon increasing pH from acid to basic conditions. Figure 2 shows the evolution of **NCC**-dye absorption spectrum upon stepwise pH raising from 4.24 to 10.93. The neutral form, **4**, has an absorption maximum at 460 nm that shifts to 540 nm in basic conditions for the formation of the deprotonated form **5**. By a sigmoidal fitting the absorbance values at the maximum versus pH (Top inset in Figure 2), the corresponding pKa could be determined which resulted equal to 9.1±0.1, higher than our previous findings (pK=8.6)[10b]. This result could be likely due to a difference in the dye environment in **NCC** compared to a superficially hydrolyzed cellulose acetate membrane in which acetate groups could be still present and affect both the local hydrophilicity and the deprotonation properties of the sensing molecule.

The loading of **1** on **NCC** could be determined by UV-vis spectrophotometry. To this purpose, the molar absorption coefficient of **1** (assumed to be a good approximation for the molar absorption coefficient of the dye grafted onto **NCC**) was determined at pH = 10.93. It resulted to be 43000±500 $M^{-1}cm^{-1}$. On the basis of this result, a loading of 0.0195 mmol of **1** per gram of **NCC** was found, which corresponded to a grafting of one molecule of **1** out of every 350 glucose units, yielding a degree of grafting (DG, number of molecules of **1** for 100 glucose units) of 0.28. This figure can be corrected to take into account that some of glucose units are not available for the functionalization namely 1) those in the inner core of the nanocrystals and 2) half of the glucose units having the hydroxy-methyl group that point toward the inside of the nanocrystal (due to the two-fold symmetry of cellulose strands). On the basis of above reported AFM measurements, an ideal crystal with 4 nm height and 40 nm width was used for the calculations, resulting into the fact that only 15% of the bulk glucose units are useful for grafting. Thus, a more likely DG is 2 (*i.e.* 2 molecules of **1** out of every 100 glucose units).

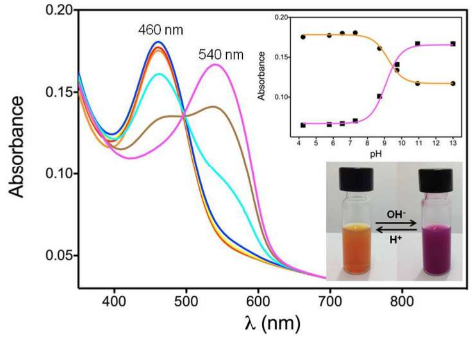

Figure 2: Evolution of UV-Vis spectrum of **NCC**-dye conjugate upon changing pH from 4.24 to 10.93 in 0.1 M phosphate buffer. *Insets*: top) Sigmoidal fittings of absorbances at 460 nm (yellow curve, dots) and 540 nm (purple curve, squares) versus pH for the determination of the pK_a of the dye; bottom) Digital picture of the suspension at pH 4.24 (yellow solution) and 10.93 (purple solution).

pH is an important parameter to be measured that allows for achieving rapid responses in many fields including clinical diagnostics, food freshness control, drinking and waste water treatment quality assessment, and bio-process monitoring. Althought electrochemical procedure is the established tool for pH measurements, optical sensing based on chemical substances that change their spectroscopical properties in response to pH offers a promising alternative. In particular, the so called optodes,[12] are made by confining a pH sensitive molecule onto a proper matrix that afterward can be used in various formats to build membranes, planar sensor spots and fiber-optic sensors.

The absorbance vs. pH curve reported in Figure 2 can be used for pH sensing using **4** as a traditional colorimetric acid-base indicator. However, the colloidal nature of **4** and its processability are instrumental to design pH-sensing devices, with tunable shape and dimensions.

The well known ability of **NCC** to form self-standing films upon water evaporation, has been exploited to produce membranes of 4 for pH sensing applications. To this aim, an aqueous suspension of **4** (1% wt, 5 mL) was casted into a polystyrene Petri dish (diameter = 5 cm) and let to slowly dry at room temperature. After two days, a uniform, yellow film (thickness *c.a*. 5-8 μm) was obtained (Figure 3, inset a) which was self-standing and transparent (*c.a.* 22% transmittance at λ=460 nm). Scanning electron microscopy (SEM) of the fractured film showed that **4** self-assembles into a characteristic multilamellar structure (Figure 3, inset a). A nearly identical morphology was observed for a film produced under the same experimental conditions but starting from unmodified **NCC** (Figure 3, inset b). Althought these membranes were quite fragile and disperded in water, they could be mechanically stabilized by attaching them to a transparent bi-adhesive tape which, afterward, was used to stick the pH-sensitive film inside a disposable polystyrene cuvette. Figure 3 shows the UV-vis spectra obtained by filling the cuvette with phosphate buffers in the 4-13 pH range. An ideal behavior was observed and fitting of absorbance vs pH curves gave an average pK value of 9.0±0.1, in agreement with the previous values found in liquid phase.

It should be pointed out that the above film was prepared from 50 mg of **4** and had a geometrical area of about 20cm^2. Since the membrane attached inside the cuvette had a surface of about 0.5 cm^2, in principle up to 40 sensors could be made at a negligible cost from each film.

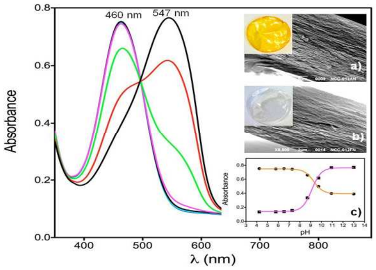

Figure 3: Evolution of UV-Vis spectrum of a membrane made from **4** (pH range from 4 to 13 in 0.1 M phosphate buffer). *Insets*: a) Digital picture and SEM image of a membrane obtained from **4**. b) Digital picture and SEM image of a membrane from **NCC**; c) Sigmoidal fittings of Absorbance vs. pH curves recorded at λ=460 nm (yellow curve, dots) and λ=540 nm (purple curve, squares) for the determination of the pKa of the dye.

The versatility of this material is such that it can be shaped into a variety of formats. As an example, a piece of the film made from **4** was taped on a plastic strip to build a pH indicator stick which proved to respond quickly and reversibly to pH changes as evident in the movie reported in the Electronic Supplementary Information section. In addition, a series of digital pictures of the stick after soaking it briefly on a buffer solution were simply taken with a cellular phone digital camera (Figure 4a). Pictures were subsequently processed with a photo-editing software to estract the information about the color of the pH stick in terms of red-green-blue (RGB) values. In particular, the green channel was the most sensitive to pH variations. A graph of green value vs. pH (Figure 4, bottom graph) has the typical sigmoidal shape and the pK value extracted by fitting was 8.8±0.1, slightly different from the above findings. That could be the results of the digital imagine technique which is less accurate than a regular UV-Vis spectrometer.

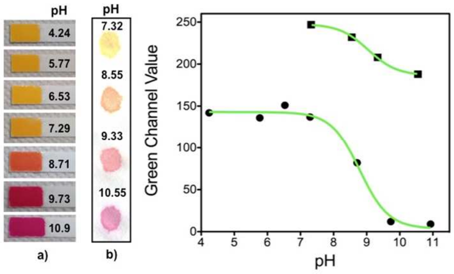

Figure 4: Plot of green channel values vs. pH plot for pH sticks (bottom graph) and spots of **4** (top graph). *Insets*: a) Digital image of pH-stick after dipping in 0.1M phosphate buffers at different pH. b) Digital image of spots of **4** on filter paper exposed 0.1M phosphate buffers at different pH**.**

Noteworthy, colloidal **4** is readily formulated as water-based ink, to be printed on supporting surfaces, with tunable pattern and dosage. In a preliminary test, 4 has been spotted on filter paper and left until complete dryness, to yield persistent stains that are not removed after extensive washing. Digital image analysis of the on-spot colorimetric response upon treatment with buffers in the pK range, (Figure 4b) confirmed the expected pH-response (Figure 4, top graph). The ink-jet printable perspective retains a valuable potential in the field of smart packaging with integrated chemical sensors for direct visual detection.

In conclusion, we reported the one step, aqueous phase synthesis of a **NCC**-dye conjugate which is amenable to the production of pH sensors in different formats. These sensors are bio-compatible, cost-effective and could be mass produced. We are currently extending the scope of the research by using a palette of acidochromic dyes which have been previously designed for optically monitoring pH in the range from 3 to 12.[13] These dyes can be used in array[10b] or in a cocktail blend by exploiting the ink-jet printing technology.[14] Finally, we are studying the application of vinylsulfone chemistry to broaden the scope of **NCC** functional materials with molecules other than azo-dyes.

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