

The pyranine-benzalkonium ion pair: a promising fluorescent system for the ratiometric detection of wound pH

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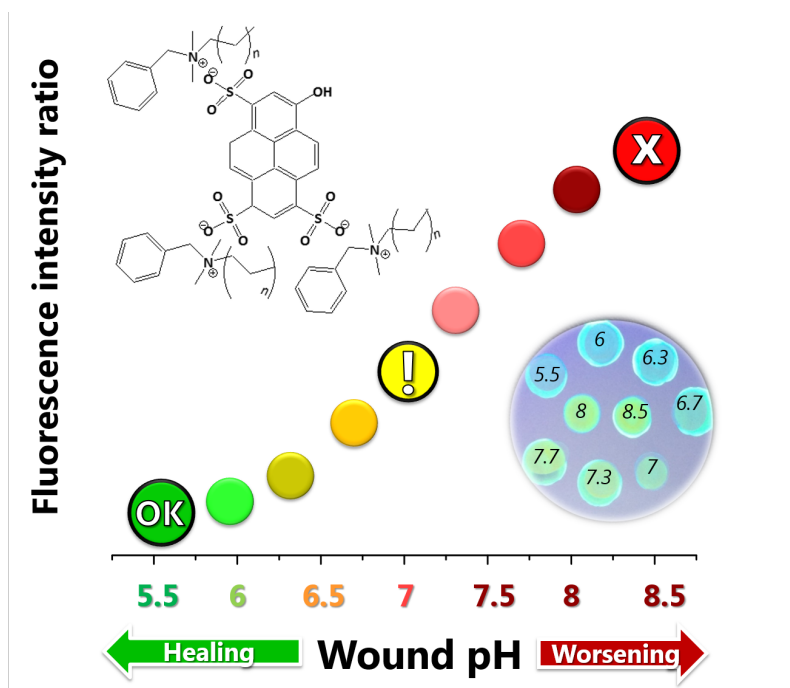
ABSTRACT

Pyranine is a non-toxic fluorescent dye, with a broad range of applications in biomedicine where it allows precise ratiometric detection of pH within the physiological pH window. Hereby, the facile synthesis, potentially interesting for commercial applications, of a novel ion pair between pyranine and benzalkonium is described. Benzalkonium, a quaternary ammonium salt with potent antimicrobial properties, makes pyranine sufficiently hydrophobic to allow its incorporation in conventional membranes and commercial wound dressings while maintaining its pH sensing performance unaffected. In conclusion, this novel fluorescent ion pair could be extremely promising for the non-invasive monitoring of wound pH.

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HIGHLIGHTS

- A novel hydrophobic ion pair of pyranine and benzalkonium is described.
- The pyranine-benzalkonium ion pair is suitable for the ratiometric fluorescent detection of wound pH.
- Ease of synthesis, the use of intrinsically non-toxic and commercially interesting precursors and antimicrobial properties make the ion pair appealing for biomedical applications.

KEYWORDS

Pyranine, benzalkonium chloride, pH sensing, ratiometric, chronic wound monitoring, antimicrobial

INTRODUCTION

Chronic wounds are a costly healthcare issue and are of increasing relevance especially in countries with ageing populations [1]. This generates a demand to improve the quality and performance of wound dressings, which would make wound care easier, more comfortable for the patient, and more cost-effective for healthcare providers. The current practice for wound care involves the manual removal of a dressing for visual inspection of the underlying wound and the sampling of exudate for *ex vivo* measurements. These are not only time-consuming tasks for the nursing staff, but more importantly, they are painful for the patient and disturb the natural healing process of the wound, moreover increasing the risk of infections. Novel concepts for improved wound dressings allow *in situ* sensing of wound parameters (including temperature, moisture, oxygenation and pH), alerting medical staff to the possible presence of infection and other healing characteristics without the need to remove the dressing [2,3]. Such smart dressings, in turn, have the potential to generate significant savings in healthcare costs thanks to reduced clinical inspection time and shorter hospital stays as a result of faster wound healing. Of course, these new dressings must also need to be reasonably cheap, as high costs would greatly limit their acceptance by clinicians.

Of all the parameters of interest for the chronic wound, pH has been indicated as the most determinant [4,5]. While the natural pH of the skin may vary from person to person, it typically fluctuates within a narrow acidic range between pH 4 and pH 6, which helps to control bacterial proliferation. Whenever an injury generates a cutaneous wound, it exposes the underlying tissue, which, as it is normally regulated at pH 7.4, can encourage bacterial adhesion and proliferation and thereby promote infection. The pH of the chronic wound, however, oscillates in a weakly alkaline range between pH 7 and pH 8, increasing the susceptibility towards bacterial infestation [6]. Such increase in pH has been proposed as a diagnostic tool to identify the onset of an infection [7]. Consequently, there is an increasing effort to develop disposable pH sensors for non-invasive wound monitoring.

To this end, pH-sensitive colorimetric dyes were the first obvious candidates to be chosen. However, since sensors based on light absorbance are not as sensitive as those based on fluorescence, pH-sensitive fluorometric dyes rapidly became the most studied. Depending on the pH, some fluorescent dyes possess different absorption maxima due to differences in their acidic (protonated) and basic (deprotonated) forms. While the emission intensity is dependent on the excitation wavelength, the emission maxima is usually unaffected by pH. Thus, it is possible to measure the emission intensities at two excitation wavelengths, and the ratio of the emission intensities becomes a function of pH. Such a “dual excitation”-“single emission” measurement

process is a highly reliable method for the determination of pH since it minimizes the impact of detrimental factors such as fluorophore concentration, photobleaching, excitation light intensity fluctuation and detector sensitivity. Examples of dyes exhibiting this kind of behavior include carboxydichlorofluorescein (CDCF), carboxynaphthofluorescein (CNF) and 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS, pyranine) [8].

We have recently developed chemical and bio-sensors based on fluorescein derivatives (CNF and fluorescein isothiocyanate FITC) for different applications. pH sensors were constructed by incorporating a FRET-forming dye pair into xerogel matrices [9,10], where FITC was used either as donor or acceptor. The system exhibited good reversibility and could be used as coating for a range of substrates, including plates, optical fibers, and textiles [9,10]. Based on that system, we developed a simpler one, containing CNF as single dye as a potential pH sensor for chronic wounds [11]. Another derivative, 2',7'-dichlorofluorescein-diacetate (H2DCF-DA), was combined with a coupled enzymatic assay to function as a bio-sensor to *e.g.*, glucose in the wound exudate [11]. However, these three systems are intensity-based and thus prone to the disadvantages mentioned above.

In the present work, we turned our attention to HPTS: it is a hydrophilic, pH-sensitive fluorescent dye with very good photostability (a property shared also by its derivatives) [12] and a broad range of applications for optical sensors [13-20]. HPTS has also been used for various biological applications, *e.g.* for the measurement of intracellular pH, thanks to its high water solubility and low toxicity [21,22]. In addition to all these favorable properties, HPTS has two different, pH-dependent, excitation wavelengths (at ~405 and ~460 nm, respectively) which correspond to an emission at ~514 nm. The pH sensitivity of HPTS arises from the OH group placed in the position 8', whose $pK_a = 7.3$ corresponds to a pH calibration range ranging from pH ~6 to ~9, adequate for biological applications and especially suitable for the monitoring of pH as bio-marker of the wound status.

However, a significant drawback for the application of HPTS in aqueous environments is its high solubility in water. To overcome this problem, we exploited the strategy originally developed by Wolfbeis [23], in which alkali counterions of hydrophilic dyes are replaced by quaternary ammonium groups with long aliphatic alkyl chains by reacting them with the respective bromide or chloride to form a lipophilic ion pair. Such an approach has been extensively used to make indicator dyes sufficiently hydrophobic to allow their incorporation into polymer and sol-gel matrices. Tetrabutylammonium bromide (TBAB), tetraoctylammonium bromide (TOAB) and especially cetyltrimethylammonium bromide (CTAB) have been the most used quaternary ammonium to form a lipophilic ion pair [23].

Benzalkonium chloride (BZKC) is a quaternary ammonium compound with relevant industrial applications as a biocide, as a cationic surfactant and as a phase transfer agent. BZKC is used as a disinfectant, detergent, and antiseptic. The mechanism of its bactericidal effect is thought to be the disruption of intermolecular interactions, which eventually cause the dissociation of cellular membrane bilayers. Because of its broad range of antimicrobial activity and its non-toxicity, it is the preservative of choice for most multiuse aqueous nasal, ophthalmic, and otic products. It is used in hand sanitizers, first aid products (to prevent infection in minor scrapes and cuts), leave-on skin antiseptics, hygienic wet wipes, and disinfectant solutions for both clinical and household use [24-26]. Moreover, it has been demonstrated that BZKC still retains its antimicrobial properties when incorporated, also by ion pairing, into different matrices [27,30].

Here we report the use of benzalkonium chloride to make form a hydrophobic ion pair with pyranine with double features as pH sensor and antimicrobial coating and we demonstrate its suitability as a fluorescent system for the ratiometric detection of wound pH.

RESULTS AND DISCUSSION

Synthesis and characterization of the pyranine-benzalkonium (HPTS-BZK) ion pair

The synthesis of the pyranine-benzalkonium ion pair was attained by mixing aqueous solutions of the dye and of the quaternary ammonium in a 1:3 molar ratio, as shown in equation 1:



A detailed description of the synthetic methodology can be found in the Supporting Information.

Figure 1A shows the ATR-FTIR spectra of three different samples, namely: pure HPTS, pure BZKC and the resulting ion pair. The most relevant absorption bands and peaks are: 3700-3000 cm^{-1} , OH stretching (for all the three samples); 3000-2780 cm^{-1} , CH_2 and CH_3 stretching (BZKC and HPTS-BZK); 1480-1430 cm^{-1} , CH_2 and CH_3 bending (BZKC and HPTS-BZK); 1250-1100 cm^{-1} , S=O stretching (HPTS and HPTS-BZK); 1100-1000 cm^{-1} , SO_3^- stretching (HPTS and HPTS-BZK); 1000 cm^{-1} , C-N stretching (BZKC) [27,28]. The formation of an actual ion pair between HPTS and BZK, rather than simply having a physical mixture, is demonstrated by the broadening of the S=O stretching band and by the blue shift of the SO_3^- stretching peaks (1010 cm^{-1} vs 1005 cm^{-1} , 1046 cm^{-1} vs 1035 cm^{-1} , 1073 cm^{-1} vs 1056 cm^{-1} for HPTS and HPTS-BZK, respectively) (Figure 1B) [27,28].

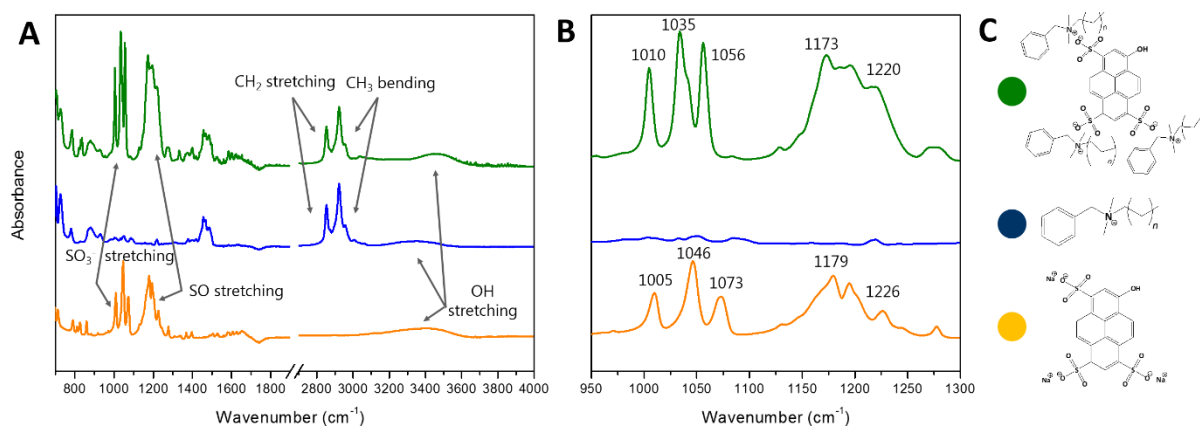


Figure 1. ATR-FTIR spectra of HPTS (yellow), BZKC (blue) and HPTS-BZK (green) ion pair (a) and its magnification on the spectral region 950-1300 cm^{-1} , b) along with their molecular structures and color legend (c).

The UV-visible absorption spectrum of the HPTS-BZK ion pair in ethanol is shown in Figure 2A. The absorption maximum of this compound is at 404 nm with an extinction coefficient $\varepsilon = 2.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, which is in accordance with reported values for pure HPTS [29]. In presence of a base, such as NaOH, the absorption maximum is shifted to 456 nm. Excitation at these wavelengths resulted in emissions at 436 nm and 514 nm, respectively (Figure 2B).

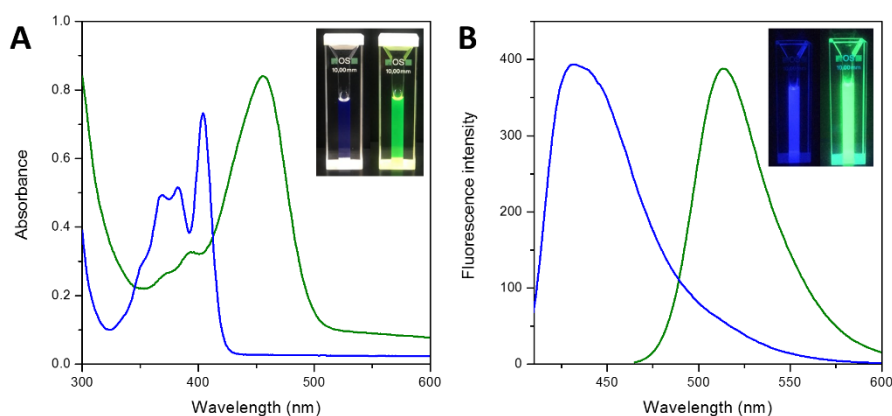


Figure 2. UV-Visible absorption spectra (A) and fluorescence spectra (B) of the HPTS-BZK ion pair in ethanol in absence (blue line) and in presence (green line) of 0.1 M NaOH. The concentrations used for the absorbance and fluorescence measurements were 0.1 mg mL^{-1} and 0.025 mg mL^{-1} , respectively.

Test of pH-responsiveness of the HPTS-BZK ion pair

Immobilization of the ion pair on a solid substrate was achieved by drop-casting 50 μL of a 15 mg mL^{-1} HPTS-BZK solution in absolute ethanol directly on poly(vinylidene fluoride) (PVDF)

membranes followed by solvent evaporation (in air or under vacuum). The morphology of the membranes was not affected by the treatment, as shown by electron microscopy images (see Supplementary information, Figure S1). Commercial cotton-based wound dressings were also evaluated as substrates to demonstrate the proof-of-concept for future application (see Supplementary information, Figure S2).

Chronic wounds, including pressure ulcers, diabetic foot ulcers and venous ulcers, exhibit a varying degree of exudate production depending on several factors (*e.g.* wound size and stage of healing) [31,32]. In general, wound fluid is rich in electrolytes and phosphate-buffered saline (PBS, pH 7.5; composition: 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl) has already been proposed to mimic wound exudate for the testing of wound pads [32]. Solutions with different pH values, ranging from 5.5 to 8.5, were prepared by adjusting the pH of the buffer with aqueous HCl and NaOH 1 M solutions. Given the high ionic strength I of phosphate buffer saline ($I = 187 \text{ mmol kg}^{-1}$, calculated using the equation $I = \frac{1}{2} \sum m_B z_B^2$, where m_B is the molality of ion B and z_B is its charge), the overall change associated by pH adjusting was considered to be negligible for our study, in accordance with other literature protocols [33]. The membranes with immobilized ion pairs were tested at various pH values by using the supra-mentioned buffer, and the emission intensity at 511 nm for excitation at both 405 and 460 nm after 1 min was recorded (Figure 3A and 3B). The ratio between the intensity of emission after excitation at 405 nm (I_{405}) and after excitation at 460 nm (I_{460}) was calculated and plotted vs. the corresponding pH value. A corresponding sigmoidal curve was obtained, which allows an efficient discrimination between different pH values in a range relevant for chronic wounds (*i.e.* 5.5-7.5).

Compared to state-of-the-art, single-use sensors, our system presents many advantages: the materials used are non-toxic and cytocompatible, the chemicals and the synthetic process themselves are easy to handle. It is noteworthy to state that ethanol is low-class toxicity solvent according to ICH guide solvent list. Moreover, one typical problem exhibited by dye-loaded pH sensors is that of leaching, *i.e.* loss of the indicator due to desorption from the matrix. Thanks to the use of a ratiometric detection approach, leaching of the dye is less likely to affect the sensor's performance. In our case, leaching could even be advantageous because it would result in a release of benzalkonium, a powerful antimicrobial agent. Therefore, such sensor attains a reproducible pH calibration.

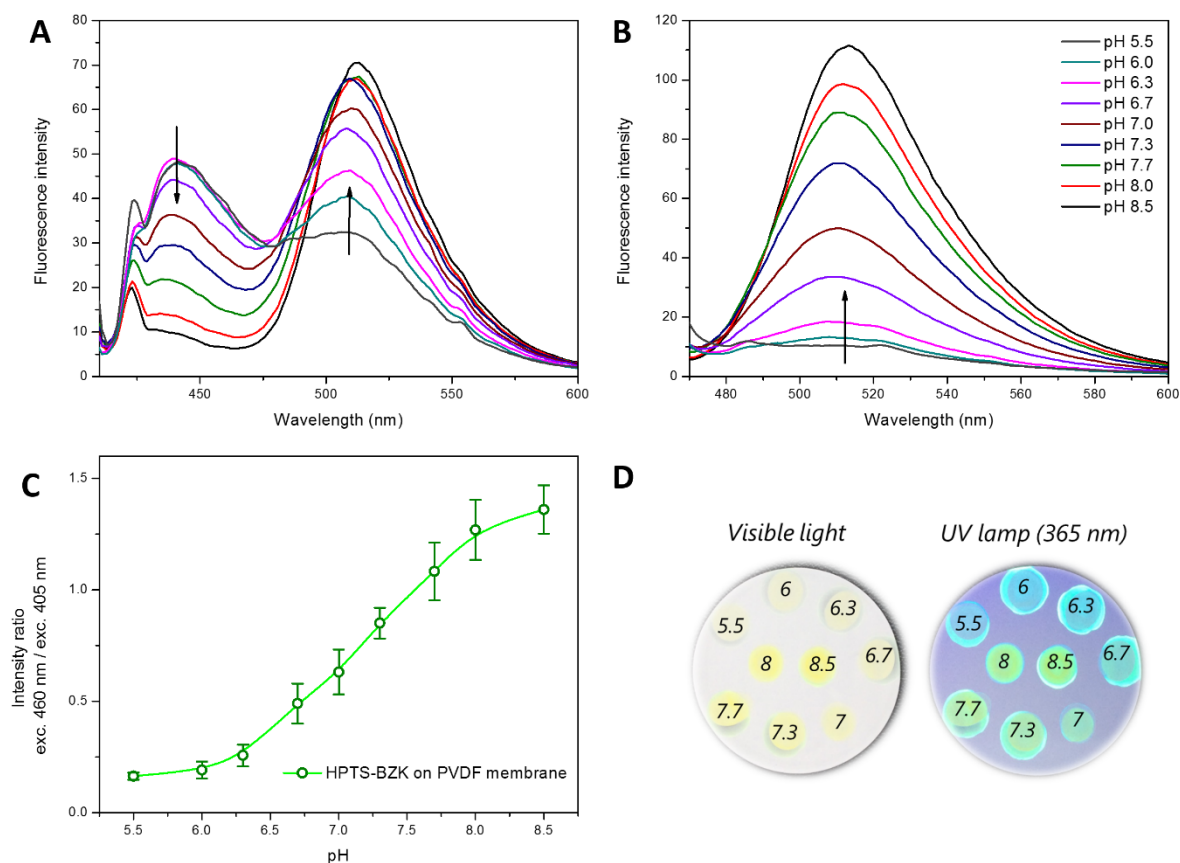


Figure 3. Emission spectra of the HPTS-BZK immobilized on PVDF membrane as a function of pH, obtained with (A) $\lambda_{exc} = 405$ nm and (B) $\lambda_{exc} = 460$ nm. The resulting calibration line (average of measurements of three different membranes) (C) is the ratio between the emission at 511 nm for two different excitation wavelength (i.e. 405 nm and 460 nm). The color change of the immobilized ion pair as a function of pH is depicted in (D) under both visible and UV light.

Test of antimicrobial properties of the HPTS-BZK ion pair

In a next step, the HPTS-BZK ion pair was assessed for its antimicrobial activity against methicillin-resistant pathogenic bacteria *S. aureus*. Since HPTS-BZK is not water soluble, it was coated onto a silicon wafer (see the Supplementary Information). After 2 h incubation of the different surfaces with *S. aureus* cells at 10^5 colony-forming unit (CFU) per mL, the HPTS-BZK coated surface killed all applied *S. aureus* cells, whereas the silicon control surface did not exhibit antimicrobial activity and allowed almost 100% recovery of the applied cells (Figure 4). Thus, the HPTS-BZK ion pair prepared in this work possesses excellent antimicrobial property.

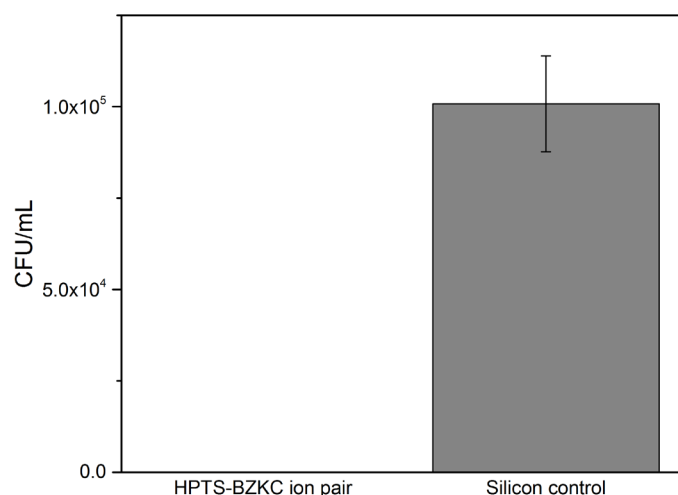


Figure 4. Antimicrobial property of the HPTS-BZK ion pair against *S. aureus*. The data generated from two independent experiments are expressed as the average \pm standard deviation (N = 6).

CONCLUSIONS

We described the synthesis of a new ion pair between pyranine and benzalkonium (HPTS-BZK) and its use in detecting the pH values in wounds as key bio-marker of wound status via colorimetric and fluorescence intensity calibration in a ratiometric mode. The well-documented non-toxicity of both precursors as well as the deposition solvent makes this sensor particularly appealing for its embedment in biomedical devices. Although the leaching of pyranine is a stringent issue in several ion pair mixtures due to the scarce resistance of the ion pair to ionic strength, the up-scalable and low cost physical entrapment in PVDF filter as well as cotton-based pads via drop-casting paves the way for a direct integration of the sensing units in commercial wound pads.

The pH calibration range is perfectly suitable for the detection of wound pH (i.e. 5.5-7.5) even in the presence of ionic buffers, such as PBS saline media. Moreover, we found that for pH values higher than 7 there is a striking change of the absorbance and the fluorescent emission color, which can be easily evaluated by using a commercial UV LED lamp. Semiquantitative results could be obtained by the aid of a smartphone camera as spectrometer [34]. This would give the patient/nurse/clinician the tools for the discrimination of the wound status, even in the absence of a canonical ratiometric detection apparatus. This would be very important especially for self-monitoring at home or for dedicated services.

In conclusion, the pyranine-benzalkonium ion pair appears to be a promising candidate for the development of point-of-care wound diagnostics sensors.

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