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Polymeric chemosensor for the detection and quantification of chloride in human sweat. Application to the diagnosis of cystic fibrosis

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We have developed a new extremely hydrophilic polymeric film suitable for the detection and quantification of chloride in human sweat directly on the skin. The film, or membrane, has chemically anchored 6-methoxyquinoline groups as chloride responsive fluorescent motifs. We have prepared the sensory material from a standard vinyl copolymer, by a convenient and easy solid-phase reaction. The sensory material has a water swelling percentage of 700%, facilitaing an inmediate detection of chloride, is reusable for at least 6 cycles and can be handled without care by unskilled persons. The initially high fluorescence of the material decrease in the presence of chloride, allowing the quantification of the chloride concentration by using the colour definition of a digital picture or a fluorimeter. The suitability of the material to perform quantitative chloride analysis of human sweat by putting it in contact with the skin offers promise for is application in the sweat test used for the diagnosis of cystic fibrosis (CF).

Introduction

The development of point-of-care (POC) diagnostics technologies has a huge potential for the advance of medical sciences.[1] Advances in microfluidics and nanotechnology are resulting in an increasing number of POC devices and platforms providing rapid, sensitive and user-friendly analyses and diagnostics with real world applications.[2,3] The possibility of using smartphones with their portability, connectivity, apps and digital cameras broadens the limits of these technologies, providing opportunities to create devices for monitoring and share health information at the point of living, remote areas and non-laboratory settings.[4] Central to this strategy is the development of responsive elements capable of being implemented into devices, enabling monitoring or early detection.[5]

Chloride is an essential electrolyte, thus quantification and monitoring of chloride concentrations in various fluids is an important bioparameter.[6] Of particular relevance is the monitoring of chloride for the diagnosis of cystic fibrosis (CF).[7] CF is a genetic disease affecting the CFTR gene resulting in alterations of the amount or functionality of the CFTR transmembrane protein. This protein is a chloride and bicarbonate selective transmembrane channel expressed in epithelial tissues, and the malfunction of this protein is responsible for the abnormal high concentration of chloride in sweat. Detection of this alteration in the so-called sweat test is the accepted clinical evidence for the diagnosis of CF.[8,9] The sweat test involves the collection of sweat induced by iontophoresis and subsequent analysis of the chloride contents. The Macroduct system, developed by Wescor, is widely used in the clinic.[10] This system measures the electrolyte concentration by electrical conductivity. There are clinical evidences that conductivity measurements constitute a reliable indicator to confirm a clinical diagnosis of CF. Since the main anions present in sweat are chloride, lactate and bicarbonate, the conductivity value can be used to obtain an estimation of the chloride concentration as demonstrated in the clinic.[11]

At present, there is no cure for CF and the pharmaceutical industry is making an intense effort to develop drugs for the treatment of this disease. There are two medicines currently approved, ivacaftor and orkambi, representing examples of the main strategies for the drug discovery in this field.[12,13] These strategies aim to enhance the transport rate of functional CFTR proteins in the membrane, potentiators, or correct defects in the production or delivering of CFTR to the plasmatic membrane, correctors. Aside from the diagnostic value, the sweat test constitutes an excellent tool to monitor the performance of these drug candidates in clinical trials. Therefore, an inexpensive, easy to use by patients at the point of living, chloride sweat test would constitute a valuable tool

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for this purpose. Targeting this goal, several methods have been implemented toward simplifying the analysis of chloride in sweat for the diagnosis of CF.[14-15] Fluorescence is a most appropriate readout for measuring and detecting this anion and very recently variations of the fluorescence of molecular sensors have been used to quantify chloride within this context. [16-18] However, these systems require the collection of sweat from the patient and manipulation of the sample with the addition reagents such as concentrated acids in order to complete the analysis.

In this work, we present a new film-shaped sensory polymer for the monitoring of chloride in human sweat by direct contact with the skin using minimal amounts of sweat. Analysis of the fluorescence response of the material under these conditions with a picture taken with a smartphone allows quantification of this anion. The inexpensive and straightforward preparation of this sensory material offers promise for the development simple POC devices for performing simple, fast and cost effective chloride sweat tests.

Experimental

Materials

All materials and solvents were commercially available and used as received unless otherwise indicated (ESI, Section S2.1).

Measurements and instrumentation

The infrared spectra of the films (FT-IR) were recorded with a JASCO FT-IR 4200 (4000-400 cm^{-1}) spectrometer. High-resolution electron-impact mass spectrometry (EI-HRMS) was carried out on a Micromass AutoSpect Waters mass spectrometer (ionization energy: 70 eV; mass resolving power: >10000). The ^{1}H and ^{13}C NMR spectra were recorded with a Varian Inova 400 spectrometer operating at 399.92, and 100.57 MHz, respectively, with deuterated chloroform as solvent. The fluorescence spectra were recorded using an F-7000 Hitachi Fluorescence spectrophotometer. Thermogravimetric analysis (TGA) data were recorded using 4–5 mg of sample under a synthetic air atmosphere and nitrogen atmosphere with a TA Instruments Q50 TGA analyser at 10 °C min^{-1}. The tensile properties of **F1** were analysed using a Shimadzu EZ Test Compact Table-Top Universal Tester.

The water-swelling percentage (WSP), which is the weight percentage of water uptake by the films upon soaking until reaching equilibrium in pure water at 20°C, was obtained from the weight of a dry sample film (ω_d) and its water-swelled weight (ω_s) using the following expression: WSP = 100 × [(ω_s-ω_d)/ω_d].

Digital pictures of the sensory film (**F2**) under 365 nm light, taken with an iPhone 5S after immersion in aqueous media with different concentrations of chloride, allowed the quantification of the chloride concentration using the "R" parameter (red) of the RGB (red, green and blue) colour model.[17,18]

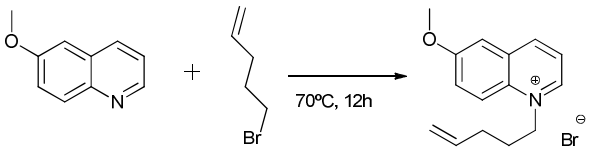
The quantum yields (Φ) were measured in *N,N*-dimethylacetamide (DMA) using quinine sulphate in sulphuric acid (0.05 M) as a reference standard.[19]

Model compound 2 synthesis

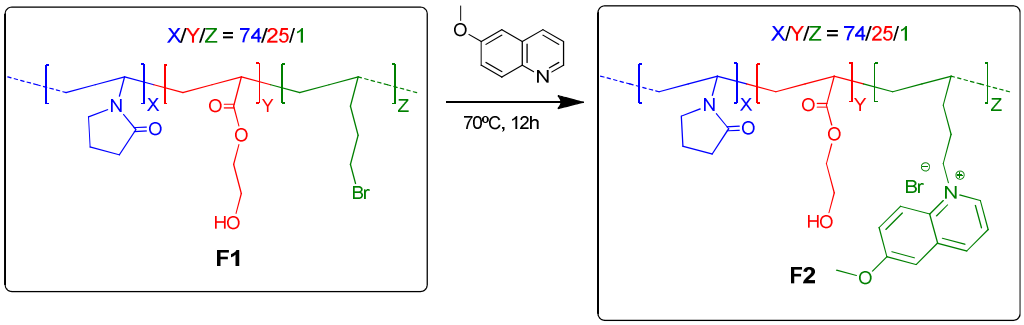
6-methoxy-1-(pent-4-en-1-yl)quinolin-1-ium bromide (**2**) was synthesized by alkylation of 6-methoxyquinoline with 5-bromopent-1-ene (see ESI, Section S1 for details).

Polymer synthesis

The final material was prepared in two steps: bulk radical polymerization of the monomers to yield polymer (**F1**), followed by solid phase reaction over the initial material furnishing fluorescent film (**F2**). The initial film or membrane (**F1**) was prepared by photochemically initiated bulk free radical polymerization from 1-vinyl-2-pyrrolidone (VP), 2-hydroxyethylacrylate (2HEA) and 5-bromo vinylpentene (**1**), at a co-monomer feed molar ratio of 74/25/1 (VP/2HEA/**1**), and using 2,2-dimethoxy-2-phenylacetophenone (1.6 wt %) as the photochemical radical initiator. The homogeneous solution of monomers was filtered off, sonicated for 5 min, injected into a 100 µm thick silanized glass hermetic mold in an oxygen-free environment, and irradiated with a UV lamp (250 w UV mercury lamp, Philips HPL-N, emission bands in the UV region at 304, 314, 335, and 366 nm with the maximum emission at 366 nm) for 12 h at room temperature.



Scheme 1 Synthesis of 6-methoxy-1-(pent-4-en-1-yl)quinolin-1-ium bromide (**2**).



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Scheme 2 Structure and schematic preparation of the sensory film **F2** from **F1**.

The modified sensory film (**F2**) was prepared by reaction of film **F1** with neat 6-methoxyquinoline in a test tube at 70ºC for 12 hours. The film was then extracted from the tube, washed thoroughly several times with acetone, and finally immersed in water for use. This reaction yielded the desired polymers with pendant covalently bonded 6-methoxyquinoline moieties (Scheme 2). Furthermore, the 6-methoxyquinoline can be reused several times, in fact, we have used the same test tube with the same 6-methoxyquinoline for preparing up to 10 pieces of 8 x 1 cm size of **F2**. Thus, the final material can be obtained, without using solvents, at a very low price.

Preparation of simulated human sweat (SHS)

For mimicking real sweat samples and performing chloride determinations on them, we have prepared aqueous solutions with the 12 of the most abundant water-soluble components of the human sweat, as described in ESI, Section S3.[20,21]

Ethical statement

We have performed all experiments with human subjects in compliance with the use and ethics of the *Hospital Universitario Marqués de Valdecilla* policy on experimentation with humans. The ethics committee of clinical experimentation of the region of Cantabria, Spain, approved this study (minute 13/2017, internal code: 2017.200) on November 16, 2017. Informed consent was obtained from the human subjects of the experimentation

Results and discussion

The fluorescence of 6-methoxyquinolinium salts is selectively quenched by halides, as previously reported.[22] This characteristic has been used to prepare halide sensitive probes and, in particular, chloride sensors in solution. These dyes are largely insensitive to pH, cations and the presence of other anions different from halides making them suitable for detecting chloride in biological fluids or cells, where bromide and iodide are essentially absent.[23] Mechanistically, the fluorescence of these dyes is quenched by a collisional mechanism and the Stern–Volmer equation can be used to determine the relationship between the fluorescence decay and the concentration of chloride. Inspired by these results, we decided to incorporate this fluorescent moiety into a hydrophilic polymeric film in order to prepare a sensory material capable of detecting chloride in aqueous solutions.

A copolymer made from non-toxic, biocompatible monomers was envisaged, thus 1-vinyl-2-pyrrolidone (VP) and 2-hydroxyethylacrylate (2HEA) were selected. Compound **2** was prepared as model and potential monomer to be employed in the copolymerization reaction. Unfortunately, it was not possible to prepare a polymeric membrane by radical polymerization with employing monomer due to radical inhibition processes.[24] Alternatively, the desired material was synthetized by solid phase reaction of the bromo appended **F1** with neat 6-methoxyquinoline (Scheme 2).

Characterization of the material

Water uptake

This is one of the most important properties of these materials, and can be easily tuned for the preparation of films with different applications. Balancing the amount of the different co-monomers according to their hydrophilic/hydrophobic character, as well as the crosslinking ratio of the material, the relative water uptake and hydrophilic character of the film can be modulated. For this application, we envisaged the need of a highly hydrophilic film in which ions can diffuse in a fast manner, allowing a rapid detection by simple contact of the hydrated film with the chloride source. **F1** and **F2** exhibited gel behaviour, with a WSP of 700%.

Thermal and mechanical analysis

4–5 mg of **F1** and **F2** samples were used for the thermogravimetric analysis (TGA) of the materials under an inert (nitrogen) or oxidizing (synthetic air) atmosphere, at a scan rate of 10 °C min^{-1}. The degradation temperatures that resulted in a 5% (T_5) and 10% (T_{10}) weight loss under nitrogen atmosphere were (T_5/ T_{10}) 312 ºC/267 ºC and 353 ºC/326 ºC, respectively. The thermal resistance of **F2** is significantly lower than **F1**´s, due to the formation of the quinolinium derivate, as is graphically depicted in ESI, Fig. S7.

Additionally, strips were cut from **F1** with 5 mm of width, 30 mm of length, and 100−120 µm of thick for the mechanical analysis. The samples were dried at 60 °C for 1 h and then immediately measured with an extension rate of 5 mm·min^{-1} and a gauge length of 10 mm. At least five samples were tested, and the average of Young's modulus, the tensile strength, and the deformation at break were 1.191 MPa, 58.59 MPa and 9 %, respectively.

CIE Chromaticity coordinates

CIE chromaticity coordinates (x and y) were calculated from the fluorescence spectra for the determination of the perceived colour of the sensory material (**F2**) upon irradiation in terms of the corresponding CIE 1931 colour matching functions (Fig. 1).[25] The practically identical CIE chromaticity coordinates for the compound (**2**) (x=0.15, y=0.08) and for the sensory membrane (**F2**) (x=0.15, y=0.09) confirms the formation of the quinolinium derivate (**2**) inside the polymer matrix. Both systems showed blue emissions.

Quantum yield

Once determined the colour of the fluorescent material, the next step is to study the fluorescence efficiency of compound (**2**) using quinine sulphate as fluorescence reference in acid media (0.05 M sulphuric acid) as we previously described.[26]

The results show that compound (**2**) has a high quantum yield (Φ = 0.63), even greater than the used reference, quinine sulphate (Φ = 0.53). This characterization gives more information about other potential applications of materials

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derived from compound (**2**), for example, luminescent converters (LUCO).[27]

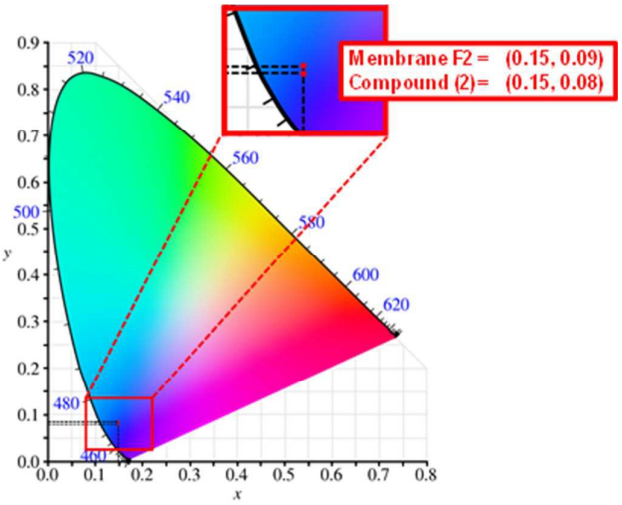

Fig. 1 CIE chromaticity coordinates (x and y) drawn on the CIE 1931 xy chromaticity diagram (red points) for the sensory membrane (**F2**) and for the compound (2) in distilled water (top and bottom points respectively). Measuring conditions: excitation slit = 5 nm; emission slit = 5 nm; excitation wavelength = 321 nm; scan speed = 1200 nm/min; the CIE 1931 xy chromaticity diagram is a public domain image downloaded from Wikipedia (http://commons.wikimedia.org/wiki/File:CIE1931xy_blank.svg).

Chloride sensing

We first decided to test the suitability of the polymeric material for the sensing of chloride. The hydrophilic polymeric membrane **F2** with pendant quinolinium units can be cut into manageable discs. The variation of the fluorescence of **F2** as function of the concentration of chloride anions can be fitted using the Stern-Volmer equation. This result implies the diffusion of water-solvated chloride inside the swelled material and the dynamic quenching of the fluorescence of polymeric system.[28] A value for the Stern-Volmer constant of 6.6·M-1 could be calculated for **F2** under these conditions (Fig. 2).The similarity of the response of the discrete molecular model 2 and the sensory polymer (**F2**) further supports the functionalization of the film with the quinolinium reporter units (ESI, Fig. S3).

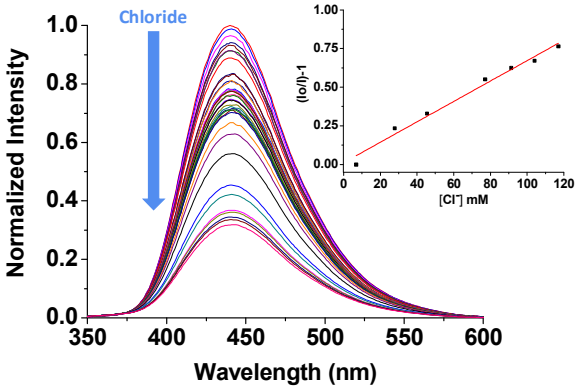

Fig. 2 Titration of chloride in distilled water using the fluorescence technique. Fluorescence spectra of a **F2** solid disc from 5 mM to 118 mM concentration of chloride. Inset: Graphical representation of "Io/I-1" vs chloride concentration (mM) to obtain the Stern Volmer constant. Conditions: Remaining time between measurements = 5 min; medium = Distilled water; excitation slit = 5 nm; emission slit = 5 nm; excitation wavelength = 321 nm; scan speed = 1200 nm/min.

Immersion of different **F2** discs in **SHS** solutions with variable chloride concentrations allowed the photograph of the progressive quenching of the initial fluorescence, and the chloride titration by using the digital colour parameters (RGB), in particular the "R" parameter (Fig. 4 and ESI, Section S5). The photographs were taken with an iPhone 5S, and the molar ratio of sensory motif was optimized to cover the full range of chloride concentration in sweat (5 mM to 100 mM).

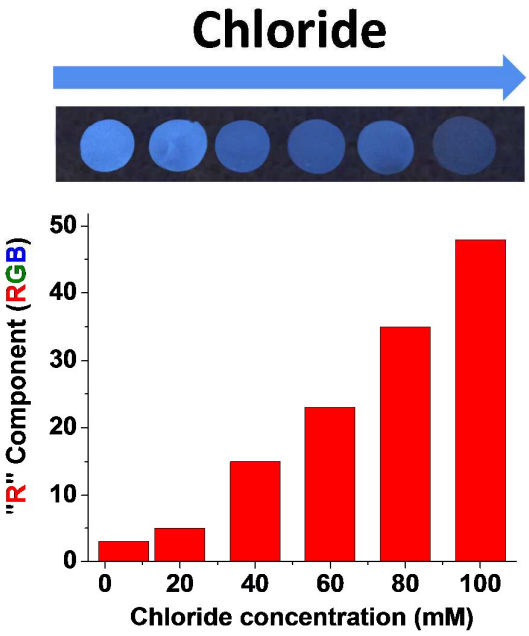

Fig. 4 Variation of R component as function of chloride concentration in **SHS** with **F2** (5.17 mM to 100 mM). A set of vials with **SHS** solutions with different chloride concentrations was freshly prepared, and one disc of **F2** (10 mm diameter) was immersed in each solution for 1 min at RT. The R parameter was obtained from the RGB parameters defining the colour of the sensory discs digital photograph. Further information in given in the ESI, Section S5.

Interference study

We next explored the potential of these films for detecting chloride in complex mixtures. An aqueous solution containing 12 of the most abundant water-soluble components of the human sweat was prepared to simulate human sweat. For this purpose, two **F2** discs were dipped for 1 minute in two chloride solutions (5.17 mM). The first prepared in distilled water and the second containing the 12 most abundant water soluble species in human sweat, at their usual concentrations in addition to chloride, including lactate, phosphate and bicarbonate (ESI, Table S2). The response of the material to chloride in both media is indistinguishable. The same response in both fluorescence and RGB analysis was observed (Fig. 3), discarding the influence of potential interferents for the

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proposed application (note that bromide and fluoride anions are potential interferents but these species are not present in human sweat in significant concentrations, so they are irrelevant for the potential application of this material).

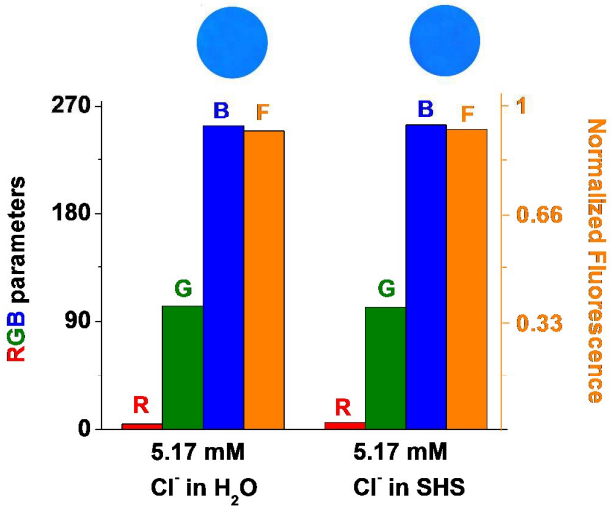

Fig. 3 Fluorescence [F, normalized fluorescence intensity at 440 nm (fluorescence intensity of pristine sensory material = 1), excitation at 321 nm] and RGB (red (R), green (G) and blue (B), RGB data of a digital photograph) analysis of the **F2** discs after dipping in aqueous solutions with chloride and chloride with a cocktail of the most abundant species in human sweat (**SHS**): The concentration of chloride in the individual and cocktail experiments, was 5.17mM. The concentration of the interferents is depicted in ESI, Table S3.

Dependence of the fluorescence of the material with the pH

We also investigated the dependence of the fluorescence of the material with the pH, specifically in the pH range 4-7 (normal pH values found in the sweat of cystic fibrosis patients).[29] The response is stable, so the material is appropriate for measuring chloride in sweat without the need of controlling or modifying the pH (ESI, Fig. S6).

Reversibility of the material

After being used for the chloride detection in **SHS** solutions, the **F2** discs were recycled for reuse by immersion in distilled water for 5 minutes (this washing process was repeated 3 times). The use-wash cycle was performed six times to demonstrate the reusability of the material (Fig. 5).

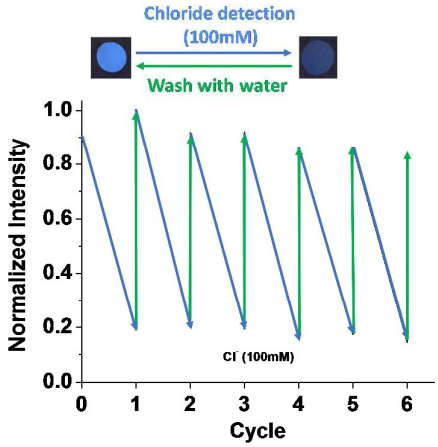

Fig. 5 Reuse of the sensory material: fluorescence reversibility measurements of a **F2** disc. Blue and green arrows represent the ON-OFF fluorescence process by consecutively dipping the disc in a **SHS** solution containing 100 mM chloride, and then washing distilled water 6 times. The digital photograph of the discs was taken under 365 nm irradiation.

Response time

The high hydrophilicity of the material is directly related with the response time. Thus, we have measured the response time of a 10 mm disc of **F2** in the fluorimeter by dipping it in a chloride solution (100 mM) in a fluorescence cuvette. The time dedicated in the preparation of the sample was about 40 seconds. After this procedure, the fluorescence of the system had reached the equilibrium, so we have determined that the response time is lower than 40 seconds (see video, ESI).

In short, our inexpensive (prepared from economical chemical/procedures and reusable), simple, and fast sensory material, can be compared with other techniques and methods for the detection of chloride anions related with the sweat test (Table 1).

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Table 1 Comparison table for different chloride sensors for the sweat test.

Sensoring system	Detection method	Detection range (mM)	Detection time	Ref.	Price
Nanoparticles & electrodes	Voltammetry	0-40	-	30	-
Potentiometric sensor	Voltammetry	10-100	40-60 (min)	14	-
Skin wipe sampling	Capillary electrophoresis with contactless conductimetric detection	Cl^-/K^+ (1.5 -12.6)	7 (min)	31	cheap
WO_3-based device	Colorimetric	30-120	Few min	32	-
Smartphone-based sensor	Visual / Fluorimetry	0.8-200	-	33	Low cost
Fluorescence indicators	Fluorimetry	10-100	10 (min)	34	2.8 €/mg
Wearable biosensors	Voltammetry	10-160	25 (min)	35	-
Polymer chemosensor	Visual / Fluorimetry	0-100	<40 (sec)	This work	0.004 €/disc

Application in the "sweat test"

In order to study the potential applicability of the material in the sweat test used for the diagnosis of cystic fibrosis we perform a simulated test as summarized in Fig. 6. 0.1 ml of **SHS** solution (10 drops of 10 microliters each) with 100 mM chloride concentration were placed on the skin of the arm of a volunteer (a researcher without cystic fibrosis). This abnormally high chloride level is expected in the sweat of a CF patient. 20 mm-diameter discs of **F2** were employed and one of the sensory discs placed over the **SHS** solution. The result was observed immediately upon irradiation with a 365 nm lamp. A clear quenching of the fluorescence was evident to the naked eye. A video showcasing this experiment is included in the ESI.

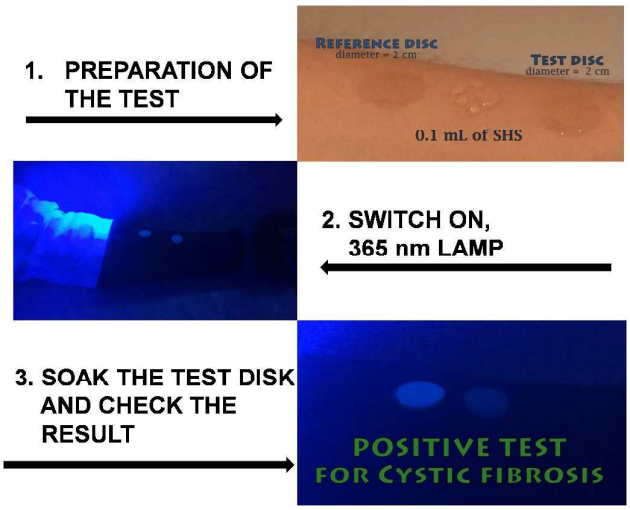

Fig. 6 Procedure followed for the chloride quantification in the sweat test.

Once the material was validated in the laboratory using **SHS**, we decided to perform real measurements in a clinic environment at the Valdecilla Hospital in Santander (Spain). In this case, the sweat is induced by iontophoresis electrotherapy in the arm of each patient during 5 minutes. After that, the sweat is collected using the Macroduct® system during 20-30 minutes, as shown in Fig. 7a and 7b. The sweat was then analysed using a sweat conductivity analyser and finally, we used the excess of sweat to perform our alternative analysis using **F2** inmediatly, taking a digital picture of the material with a smartphone and using a white reference of PTFE to homogenize all the measurements (Fig. 7c and ESI, Section S2.2). The sweat of 14 patients (9 CF diagnosed) of different ages and gender was analysed by the RGB procedure (Table 2), and the results are shown in Fig. 8. The variation of the R parameter is well correlated to the measurements obtained with the instrumentation used in the clinic, supporting the usefulness of our method to perform real sweat tests.

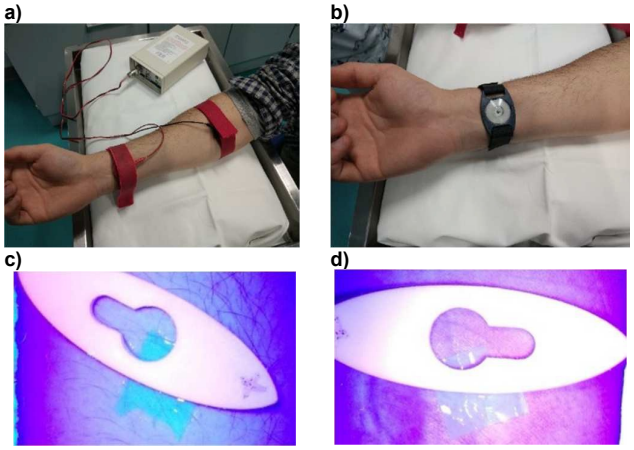

Fig. 7 (a) Inducement of sweat by iontophoresis electrotherapy. (b) Conventional analysis. Sweat recollection using Macroduct® to perform the chloride analysis in the Sweat-Check conductivity analyzer (Model 3120). (c) & (d) Analysis using our sensory material. Digital photo of arms (**c**-control, **d**-patient), white reference of PTFE and **F2** in touch with the sweat.

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Table 2 The table shows the age, the gender, the chloride concentration (obtained by conductivity analysis) and the R parameter (obtained from the RGB parameters defining the colour of the sensory discs digital photograph) of each patient.

Number	Patient	Age (years)	Gender	[Cl⁻]	R Component
1	healthy	54	F	40	78
2	CF	29	M	132	148
3	healthy	31	F	66	122
4	healthy	51	F	14	45
5	CF	48	F	78	132
6	CF	8	F	96	146
7	CF	38	M	124	146
8	healthy	14	F	43	83
9	CF	5	M	70	130
10	CF	25	M	120	147
11	CF	31	F	28	71
12	CF	46	F	11	40
13	healthy	15	M	42	91
14	CF	43	M	59	110

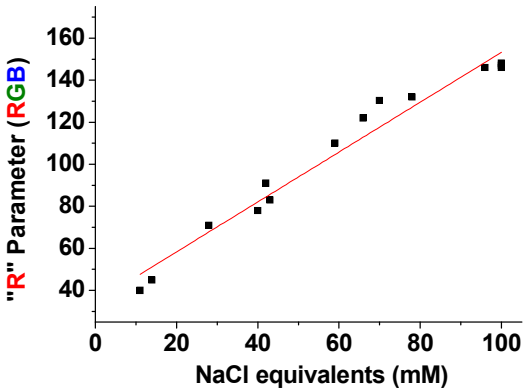

Fig. 8 Correlation between the results using conductivity (conventional analytical technique), used to control the concentration of chloride in patient sweat, and the R parameter (obtained from the RGB parameters defining the colour of the sensory discs digital photograph taken directly on the arm of the patient). Results from 14 persons (9 patients and 5 control).

Conclusions

We have prepared fluorescent film-shaped polymers with pendant 6-methoxyquinolium moieties. This inexpensive material probed appropriate for the rapid detection and quantification of chloride in complex aqueous media (water having a number of chemical species) as well as human sweat. Remarkably, the chloride quantification in sweat can be carried out just by placing the film on the skin of a patient and analysing a picture taken with a smartphone. A good correlation between the fluorescent response of the material and the conductivity measured under clinical conditions in both cystic fibrosis and non-cystic fibrosis patients was obtained. These results bode well for the application of these films in a point-of-care (POC) device for monitoring chloride concentration in biologically relevant samples. The implementation of a device for analysing chloride concentration in the sweat test aiming to monitor chloride concentration in sweat of cystic fibrosis patients is currently underway in our laboratories.

Acknowledgements

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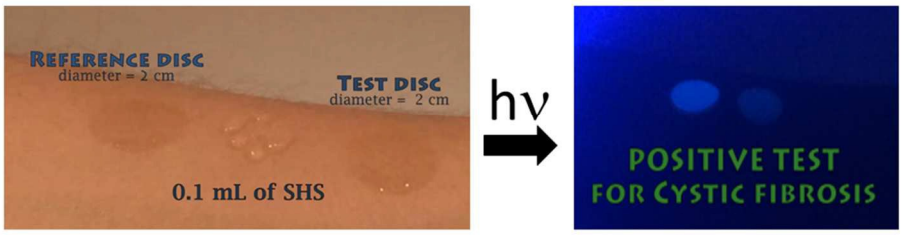

Chloride determination in human sweat

275x190mm (96 x 96 DPI)