Polymer Microarrays for the Discovery and Optimization of Robust Optical Fibre-based pH Sensors

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ABSTRACT: Polymer microarrays were utilized for the high-throughput screening and discovery of optimal polymeric substrates capable of trapping functional ratiometric fluorescence-based pH sensors. This lead to the identification of poly (methyl methacrylate-co-2-(dimethylamino) ethyl acrylate) (**PA101**) that allowed, via dip coating, the attachment of fluorescent pH sensors onto the tip of optical fibres that exhibited robust, rapid and reproducible sensing of physiological pH's.

KEYWORDS: polymer microarray, high-throughput screening, optical fibre, pH sensor

INTRODUCTION

Optical fibre-based pH sensors have a wide range of applications in both environmental and biomedical scenarios offering the advantages of miniaturization, geometrical flexibility,

real-time and on-line detection, immunity to stray electrical fields or radiation sources, biocompatibility and long-term analytical stability.[1-5] Various optical fibre-based pH sensors have been designed based on a variety of optical modalities, such as absorbance[6], fluorescence[7-9], surface plasmon resonance[10] and surface enhanced Raman spectroscopy[11,12]. Fluorescence-based pH sensors have multiple advantages over other sensor types such as high sensitivity and simplicity of operation thus occupying a prominent place amongst optical fibre-based pH sensors.[13,14]

A simple single fluorophore based pH sensor can be adapted to a more robust ratiometric type approach, that removes sensitivity to decreases in sensor concentration due to possible leaching, photo-bleaching, and/or fluctuations in the intensity of the light source or variations in light attenuation such as through an optical fibre.[4,5,15] Attachment of the pH sensor is a key-step in the development of any optical fibre-based sensor, and will often dictate the characteristics of the sensor. Multiple methods for sensor immobilization have been reported, with polymers widely used, [8,9, 16-20] however such methods often lack robustness, with high levels of leaching giving rise to poorly robust sensors.

Polymer microarrays are a high-throughput discovery platform consisting of hundreds to thousands of individual polymers spotted onto a glass slide that allows the parallel screening of libraries of polymers. These polymers have typically been interrogated with a variety of cells, with subsequent high-content screening allowing the identification of specific polymers that bind or otherwise modulate cellular function. Indeed polymer microarrays have been used to identify polymers that control stem cell differentiation or drive bacteria repellence.[21-29] In this work, for the first time, the polymer microarray technique was used to screen for polymers that display optimal performance for both sensor immobilization and pH responsiveness.

In this work, a fluorescence ratiometric detection platform, employing 5(6)-carboxyfluorescein and 5,10,15,20-Tetrakis (pentafluorophenyl)-21H, 23H-porphine

palladium (II) (as the reference dye) were implemented for pH analysis with polymer microarrays used for identifying a lead polymer that had the potential to physically entrap sensor molecules, such as fluorescent probes, displayed pH sensitivity, as well as having the ability of being dip coated as a polymer film to allow the sensor to be deposited onto the tip of an optical fibre. The fabricated ratiomatric sensors showed them to be both sensitive and rapidly responding.

EXPERIMENTAL PROCEDURES

Materials and instrumentation. All chemicals were of analytical grade and used without further purification unless otherwise stated. 5(6)-Carboxyfluorescein and 5,10,15,20-Tetrakis (pentafluorophenyl)-21H, 23H-porphine palladium (II) (abbreviated here as PdTFPP), monomers for polymer synthesis, and aminoalkylsilane functionalised glass slides (Silane-prep) were purchased from Sigma-Aldrich. Well plates were purchased from Nunc. **PA101** (Mw 226KDa, PDI 1.614) was synthesised by free-radical polymerization (see ESI†). 100 mM Sodium phosphate buffers were made up using appropriate ratios of monobasic and dibasic sodium hydrogen phosphate buffers to cover the pH range from 6.0 to 8.0. pH 4.0 citric acid/sodium hydroxide/sodium chloride buffer solution and pH 10.0 sodium carbonate-sodium bicarbonate buffer solution were purchased from Sigma-Aldrich. Graded index multi-mode optical fibre (core diameter 200 μm, outer diameter 246 μm) with a numerical aperture[30] (NA) of 0.3 was fabricated by the Centre for Photonics & Photonic Materials, University of Bath and was used in all optical sensing experiments.

GPC was conducted on an Agilent 1100 GPC equipped with 2 X PLgel MIXED-C columns ($2\times10^2 – 2\times10^6$ g/mol, 5 μm) with a guard column (5 μm) and an RI detector, eluting with DMF containing 0.1 % w/v LiBr at 60 °C at 1 mL/min and pre-calibrated using polymethyl methacrylate standards. NMR spectra were recorded on a Bruker AVA500 spectrometer and IR analysis on a Bruker Tensor 27 spectrometer. Morphological characterization of the

deposited polymer films was carried out using a Leica DMI 5000 M microscope and a scanning electron microscope (Philips XL30CPSEM).

Fluorescent images of the polymer film on the tip of the fibre were taken using a Zeiss Axiovert 200 M inverted microscope. Using Matlab the intensity images of the polymer film were processed and the fluorescein distribution analysed. The pH of the buffer solutions were measured using a glass-electrode pH meter (Mettler Toledo). The 485 nm laser was from PicoQuant (LDH-D-C-485) and was combined with a pulsed laser driver (PDL 800-D, Picoquant). A waveform generator (Keithley Model 3390) was used to synchronize the laser and the spectrometer in the optical fibre sensor system. Fluorescence spectra were captured on a spectrometer (Ocean Optics USB2000+VIS-NIR-ES) with all other optical components purchased from Thorlabs.

Preparation of polymer microarrays

Preparation of polymer solutions for printing. The polymer solutions (1% w/v) printed were a selection of a previously reported polymer library with 121 polyacrylates/acrylamides[31] mixed with three different concentrations of 5(6)-carboxyfluorescein (0.0025%, 0.01% and 0.04%, respectively) in *N*-methylpyrrolidone (NMP). Wells of a 384-microwell plate was filled with 25 μl of these polymer solutions.

Printing polymer microarrays using a contact printer. Aminosilane-treated glass slides (silane-prep, Sigma) were dip-coated with an agarose solution (1% v/v in water at 60 °C) before drying at room temperature (24 h).[32] The polymer solutions were printed onto the agarose-coated slides using a contact printer (QArraymini, Genetix, UK) with 32 aQu solid pins (K2785, Genetix). The printing conditions were 5 stamps per spot, with a 100 ms inking timing and a 200 ms stamping time and the printed slides were dried in a vacuum oven at 45 ^{o}C overnight. 4 replicate microarray slides were fabricated and used in the work.

Polymers microarray screening. The microarrays were placed in a 4-well rectangular chamber and incubated at pH 4.0 with a citric acid/sodium hydroxide/sodium chloride buffer solution for 15 mins. After incubation, the slides were imaged using a Bioanalyzer 4F/4S fluorescence scanner (LaVision Biotec), washed (x3 times with deionised water), incubated at pH 10.0 sodium carbonate-sodium bicarbonate buffer solution (15 mins) and imaged again. The fluorescence intensity of each polymer spot was quantified using ImageJ and the fold-change in intensity (from pH 4.0 to pH 10.0) determined.

Preparation of the sensors

Preparation of the coating solution. Two sensing solutions were prepared. The single-wavelength emission sensor was prepared by dissolving 50 mg of **PA101** with 16 mg of 5(6)-carboxyfluorescein in THF (1 mL). The ratiometric sensor mix consisted of 5(6)-carboxyfluorescein/PdTFPP prepared by dissolving 50 mg of **PA101**, with different proportions of 5(6)-carboxyfluorescein and PdTFPP (ranging from 1.95:1 to 195:1) in THF.

Probe fabrication. Fibres were cut to a length of 1 m, with removal of a 5 cm length of coating from each end. All fibres were cleaved with a fibre cleaver at each end of the fibre (~ 1 cm). After incubation of one end of the optical fibre in NaOH (1 M) overnight, the tips were washed with water and acetone. The fibre sensors were prepared by manually dipping the fibre tips into the coating solutions for 1 min and then withdrawing slowly (2 mins at a rate of 1 cm/min) to maintain constant polymer film deposition. In order to reduce breakage of the fibre/probe, it was threaded through a glass capillary (inner diameter 0.5 mm, length 10 cm) and fixed in place using blu tack (Bostik, UK) and stored in the dark.

Measurements with the optical fibre sensor

pH measurements. The detection system consisted of a laser system, a spectrometer, a waveform generator as the sync-signal generator, an optical coupling and collection system

which was based on the same arrangement as for a standard epi-fluorescence system (see **Figure 1**).

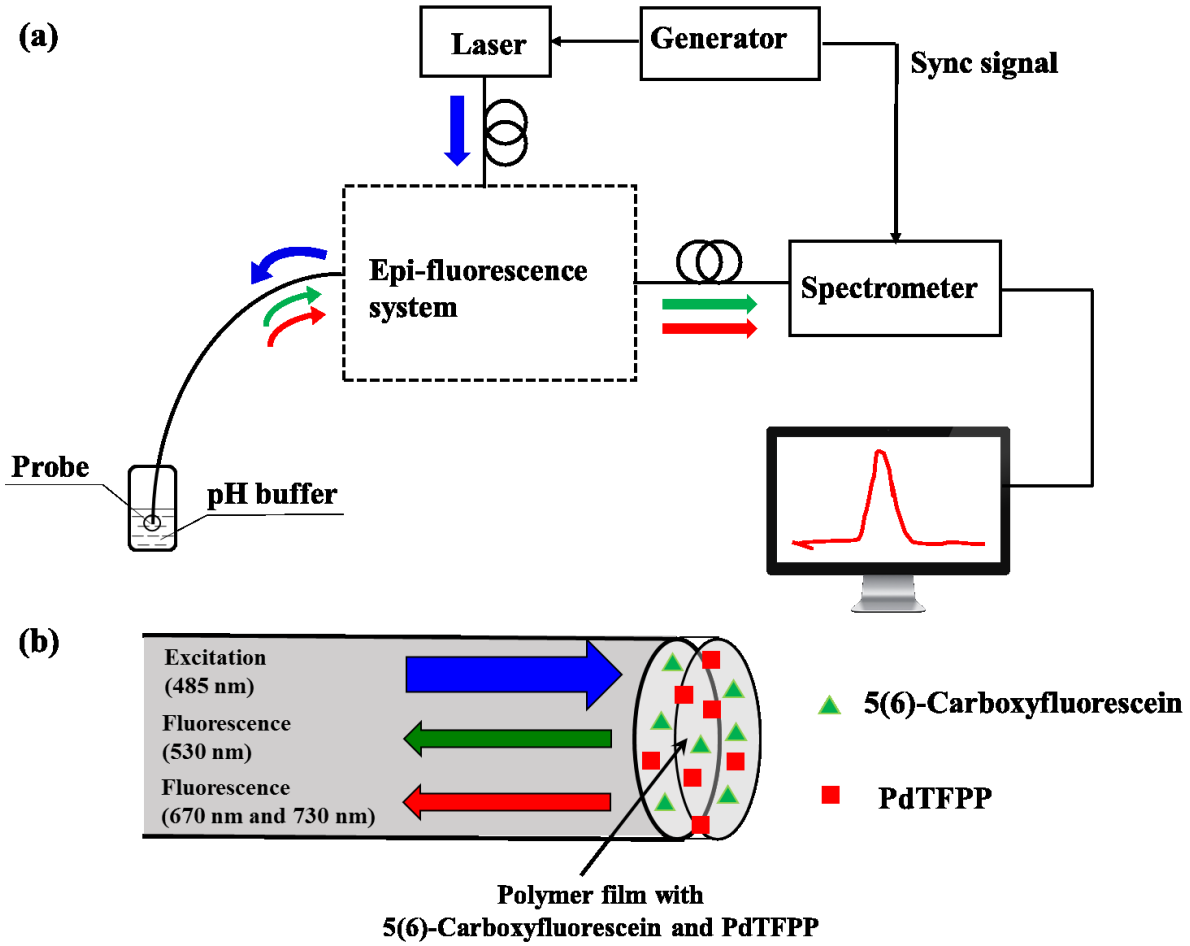


Figure 1. (a) The experimental setup of the optical fibre-based pH sensor. The pH sensitive, distal tip of a single-core optical fibre was dipped into buffers of known pH with a 485 nm laser used for excitation. The intensity of the emitted light was detected using a spectrometer via an epi-fluorescence system (see SI); (b) The distal tip of the sensor containing a mixture of 5(6)-carboxyfluorescein (green) and PdTFPP (red) to allow ratiometric measurments of pH.

The excitation light (485 nm) was launched from a single mode fibre, which then passed through the epi-fluorescence system (see **Figure S3**) into the optic fibre probe. The fluorescent components of 5(6)-carboxyfluorescein fluoresced in the green (520 nm) with increasing

intensity with pH, while PdTFPP acted as the reference dye, fluorescing in the red (two peaks at 670 nm and 730 nm) (**Figure 1**). The fluorescent light emitted from the optical probe passed through the epi-fluorescence system, and was coupled into a multi-mode fibre where it was then directed to the spectrometer. By measuring the area under the curve (AUC) for the ratio of 5(6)-carboxyfluorescein and PdTFPP, the pH of the buffers were measured and used as references.

All fluorescence measurements were conducted using a 485 nm laser (1 μW) in combination with a pulsed laser driver (PDL 800-D, Picoquant), and the fluorescence spectra recorded with an integration time of 100 ms. The probe tip was immersed into the pH buffer for 1 min before each measurement. After each pH measurement, the tip was moved to another pH buffer (pH measurements were recorded randomly and not sequentially). All measurements were carried out in the dark at room temperature (25 ^{o}C).

Reversibility. The immersion buffer was changed from pH 6.0 to pH 8.0 and back five times with emission spectra collected following excitation.

Response time measurements. The response time of the sensor was analyzed by measuring changes in the ratio of AUCs between 5(6)-carboxyfluorescein and PdTFPP, following the transfer of the probe tip from solutions at pH 6.0 to pH 8.0 and back to pH 6.0 with continuous illumination at 485 nm during the whole process, while emission spectra were recorded every 100 ms for responsive time measurements.

Photo-bleaching. The probe tip was immersed in pH 7.4 phosphate buffer and continuously illuminated by the 485 nm laser for 1 hour (1 μW), while emission spectra were recorded every 100 ms.

Leaching. The probe tip was placed in a vial containing 0.5 mL of pH 7.4 phosphate buffer for 90 mins and analysed every 5 mins, with emission spectra collected following excitation.

RESULTS AND DISCUSSION

Hit polymer identification. A high-throughput screening strategy was used to identify hit polymers that allowed efficient trapping of 5(6)-carboxyfluorescein while allowing pH sensing. The polymer-library was printed with each polymer printed in quadruplicate (**Figure 2**) with three different concentrations of 5(6)-carboxyfluorescein. The fluorescence of the polymers entrapping the dye were captured by fluorescence microscopy (representative images of the polymer microarrays shown in **Figure 2). See Table S3** for the details of the polymer microarray screening and the fold increases in fluorescence.

Due to the differences in the properties of the different polymers and the trapping and losses of the dye, the fluorescent intensity of each spot varies - with the high-throughput assay allowing the trapping ability and fluorescent responsiveness all polymers to be rapidly screened.

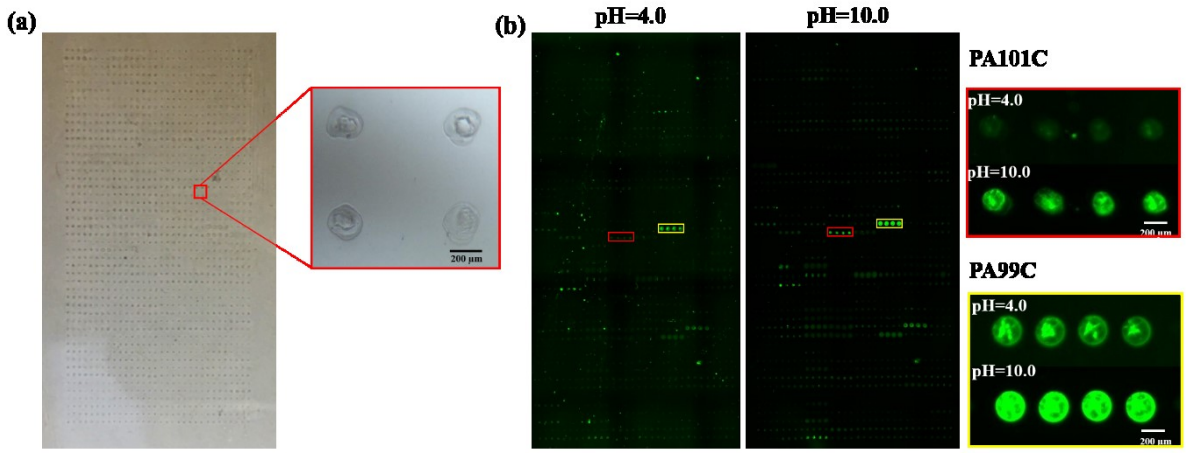
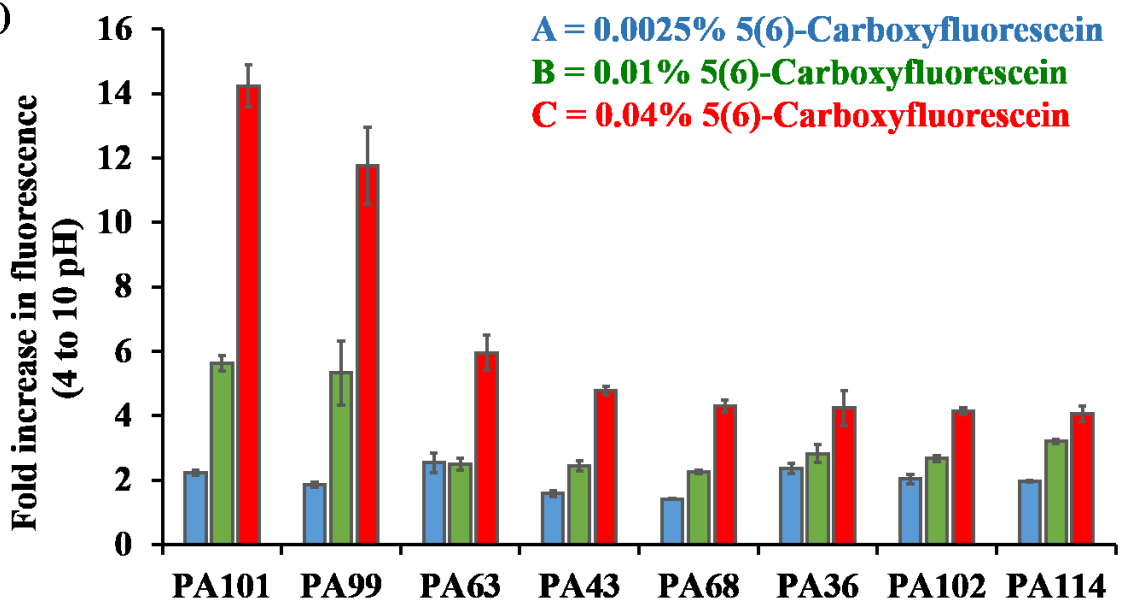


Figure 2. Images of the "sensor" polymer microarray. (a) The polymer microarray was printed with 121 polymers (each printed in quadruplicate with each polymer printed with three different concentrations of 5(6)-carboxyfluorescein in a 32 x 48 array. Inset magnification of four of the polymer features. (b) The corresponding fluorescent images for the polymer microarray after incubation in pH 4.0 and pH 10.0 buffers. The two top polymers (PA101 and PA99) are highlighted by the red and yellow boxes, respectively. The amount of 5(6)-carboxyfluorescein used was 0.04% w/v in NMP.

Figure 3 illustrates the properties of the top 8 polymers with respect to their dye-trapping abilities and pH sensitivities. The polymers mixed with low concentration of 5(6)-carboxyfluorescein (0.0025% and 0.01%) displayed low variations in fluorescent intensity with pH, whereas when the concentration of 5(6)-carboxyfluorescein was increased to 0.04% the polymers showed larger changes between pH 4.0 and pH 10.0. Two polymers, **PA99 (**poly (methyl methacrylate-co-2-(diethylamino) ethyl acrylate)**)** and **PA101 (**poly (methyl methacrylate-co-2-(dimethylamino) ethyl acrylate)) displayed the best pH sensitivities (fold increases in fluorescence of features reached 11.8 and 14.2 from pH 4.0 to 10.0). **PA99** exhibited the best dye-trapping ability (the fluorescent intensities of the **PA99C** features were the strongest), but **PA101** displayed more obvious changes between pH 4.0 and 10.0 (see **Figure 2**). In short, **PA101** had the most ideal properties with respect to pH sensitivity and trapping and was thus selected as the coating polymer for the optical fibre sensors.

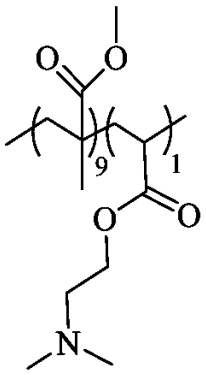
(a)



(b)

Polymer	Monomer 1	Monomer 2	Ratio of Monomers
PA101	MMA	DMAEA	90:10
PA99	MMA	DEAEA	90:10
PA63	HEMA	DMAEMA	50:50
PA43	MEMA	BAEMA	50:50
PA68	HEMA	DMAPMAA	70:30
PA36	MEMA	DMAEMA	70:30
PA102	MMA	DMAEA	70:30
PA114	MMA	GMA	50:50

(c)



PA101

Figure 3. (a) Fold increase in fluorescence for the top 8 polymers identified from the high-throughout screen. (b) Composition of the top 8 polymers. (c) Structure of the hit polymer PA101. MMA: methyl methacrylate; DMAEA: dimethylaminoethyl acrylate; DEAEA: 2-(diethylamino)ethyl acrylate; HEMA: 2-hydroxyethyl methacrylate; DMAEMA: 2-(dimethylamino)ethyl methacrylate; BAEMA: t-(butylamino)ethyl methacrylate; DMAPMAA: N-[3-(dimethylaminopropyl)] methacrylamide; GMA: glycidyl methacrylate

Properties of the polymer film. To analyse the distal surface of the coated optical fibre probe scanning electron microscopy (SEM) and optical microscopy were employed. Comparing the SEM images of the fibre before and after dip-coating (**Figure 4**) showed that the optical fibre was covered with a layer of polymer. The optical microscopy images of the sensor coated onto the tip of an optical fibre showed that the polymer film was attached onto the end of the fibre, and both 5(6)-carboxyfluorescein and PdTFPP were trapped in the polymer film. Calculating the averaged intensity along the radial of the image for the polymer trapping 5(6)-carboxyfluorescein showed a gradient-like distribution with greater fluorescence intensity at the edges of the fibre (see **Figure 4**).

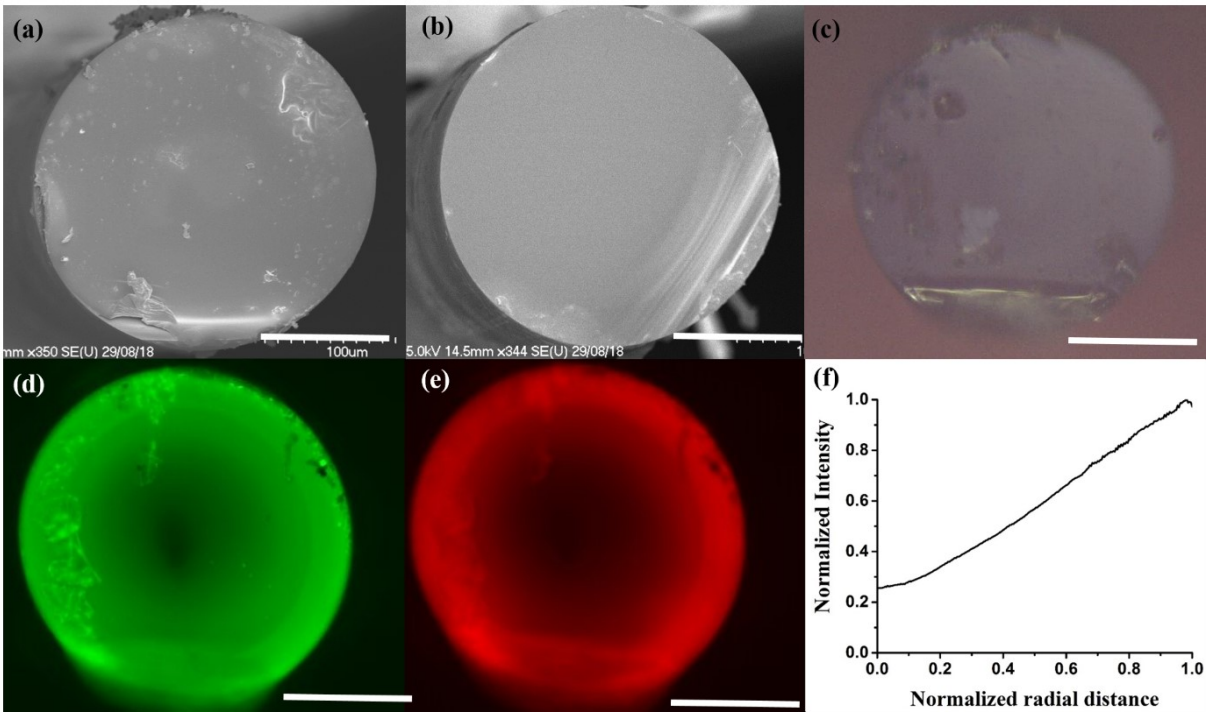


Figure 4. (a) and (b) SEM images of the distal surface of the coated optical fibre and the uncoated optical fibre respectively. (c) White light microscope image of the polymer coated onto the tip of the optical fibre (20 × objective). (d) and (e) Images for the polymer trapping 5(6)-carboxyfluorescein (ex: 485/20; em: 530/20) and PdTFPP (ex: 628/40; em: 692/40), respectively. (f) The average radial intensity plot of the fluorescent image of (d). Scale bars = 100 μm.

Properties of the sensor

pH measurements using the optical fibre sensors. 5(6)-Carboxyfluorescein has previously been used as an optical pH sensor[33], thus the single-wavelength emission sensor based on 5(6)-carboxyfluorescein was initially fabricated and analysed (see **Figure 5**). As expected the fluorescence intensity of 5(6)-carboxyfluorescein increased with increasing pH, and the area under the curve (AUC), in the wavelength range from 510 nm to 600 nm, showed the greatest pH sensitivity. However, because the single-wavelength emission sensors are vulnerable to intensity fluctuations in the excitation source or changes in emission collection efficiency, there were variations between the replicates. In order to minimize this effect, a ratiometric approach was developed with PdTFPP selected as the reference dye. Importantly, both of 5(6)-carboxyfluorescein and PdTFPP were excited by the same 485 nm laser with 5(6)-carboxyfluorescein producing an emission centred at 530 nm, with the PdTFPP producing dual emissions centered at 670 nm and 730 nm (See **Figure S4**). Both were efficiently trapped by the polymer **PA101**.

The emission spectrum of the probes trapping different ratios of 5(6)-carboxyfluorescein and PdTFPP were recorded in order to optimize the sensor mix (See **Figure S4**) with the optimal ratio to produce a robust sensor being a 39:1 molar ratio mixture of 5(6)-carboxyfluorescein/PdTFPP in the dip-coating solution. In the polymer film, the fluorescence emission of 5(6)-carboxyfluorescein at 520 nm is quenched by the proximal PdTFPP moiety. Lower ratios (less than 20:1) of 5(6)-carboxyfluorescein to PdTFPP resulted in very strong PdTFPP emission, with small emission intensity of 5(6)-carboxyfluorescein observed. With a ratio of 195:1, a strong 5(6)-carboxyfluorescein emission was observed that overwhelmed the intensity of the PdTFPP.

The normalized emission spectra of the dual 5(6)-carboxyfluorescein/PdTFPP sensor excited at 485 nm at various pH's are shown in **Figure 5**. The fluorescence intensity of 5(6)-carboxyfluorescein thus still increases with increasing pH but using the ratio of the two dyes with integration of the wavelengths of 5(6)-carboxyfluorescein (from 510 nm to 634 nm) and the PdTFPP (from 635 nm to 800 nm) provides standardized pH measurements between pH 6.0 to 8.0 (see **Figure 5**).

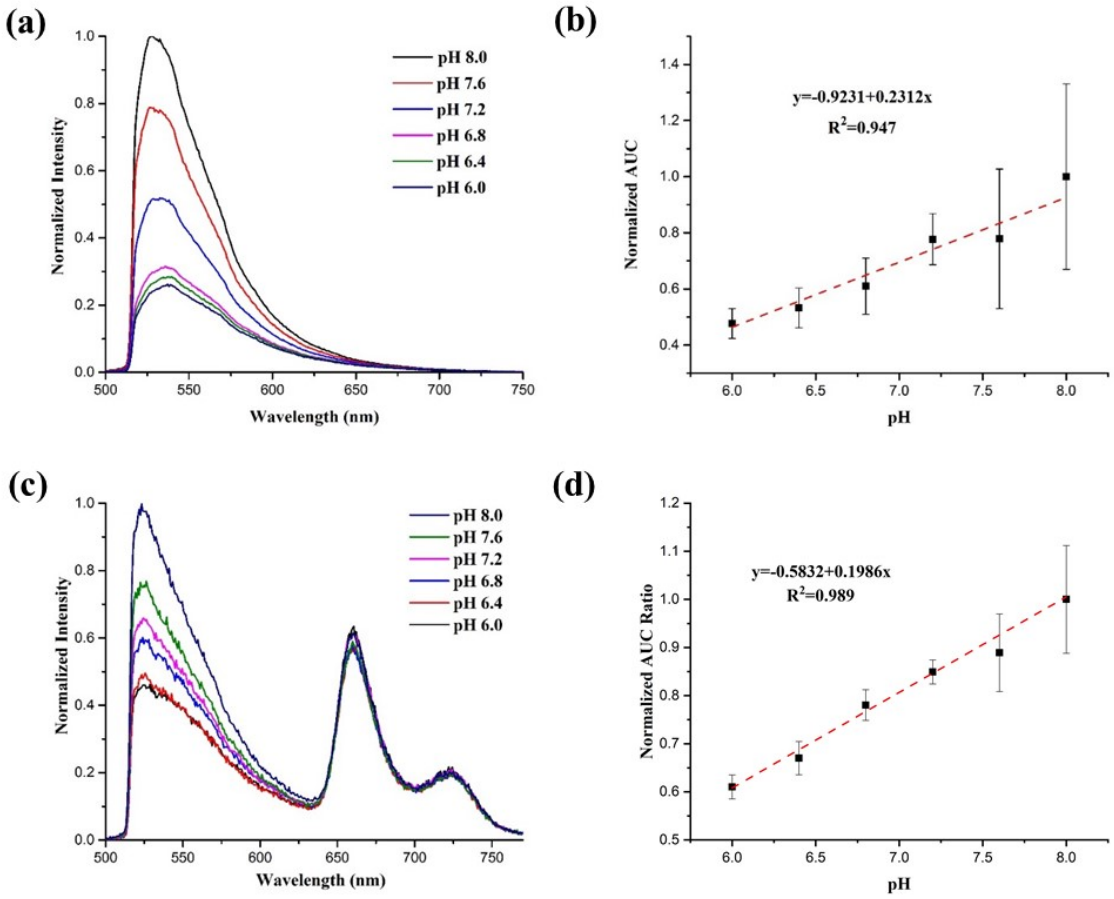


Figure 5. pH analysis of the 5(6)-carboxyfluorescein and the 5(6)-carboxyfluorescein/PdTFPP sensor between pH 6.0 and 8.0 at 0.4 pH intervals. (a) Normalized emission spectra of the 5(6)-carboxyfluorescein sensor recorded with an integration time of 100 ms. (b) Variation of the normalized area under the curve (AUC) with respect to pH in the range 6.0-8.0. The error bars represent the standard deviation of the mean over 3 replicate measurements. (c) The normalized emission spectra of the 5(6)-carboxyfluorescein/PdTFPP sensor recorded with an integration

time of 100 ms. (d) Variation of the normalized AUC ratio with respect to pH in the range 6.0-8.0. The error bars represent the standard deviation of the mean over 3 replicate measurements.

Reversibility and time-response. The pH was changed from 6.0 to 8.0 and back five times to measure the robustness of the system (**Figure 6**) and demonstrated that the sensor showed good reversibility. The response to pH was rapid within the time frame of the experimental set-up with the calculated response time being 10.0 ± 0.4 s and 16.2 ± 1.4 s when the buffer solutions were changed from pH 6.0 to 8.0 and from pH 8.0 to 6.0 respectively (**Figure 6**, n = 3).

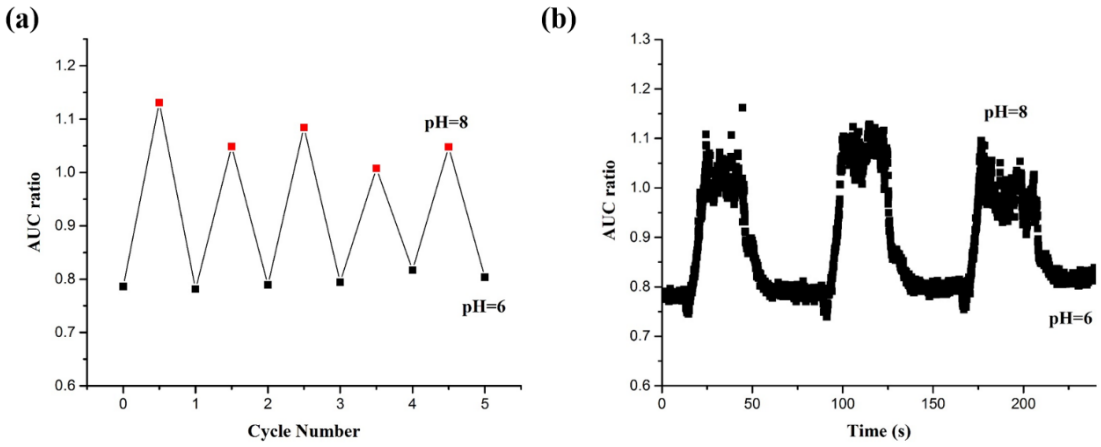


Figure 6. (a) Reversibility test of the sensor between pH 6.0 and pH 8.0; (b) Time response of the sensor measuring changes in fluorescent intensity following movement of the sensor between solutions at pH 6.0 and 8.0 repeatedly.

Photo-stability. To assess the photo-stability of the sensor, it was immersed in PBS at pH of 7.4 with continuous laser illumination (485 nm, 1.0 μW) applied to deliberately maximize damage to the fluorophores. This caused both dyes to show significant photo-bleaching during the measurement (See **Figure 7** (a)). Due to this effect, the exposure time was set to 100 ms and the triggered system was employed to ensure that laser illumination was only used for the

duration of the measurement (the integration time) to avoid unnecessary photo-bleaching between measurements.

Leaching or dye washout was even less significant than photobleaching. Leaching was thought possible since the dye molecules were only physically entrapped in, and not covalently linked to, the polymer, although this was an integral part of the microarray screen. To determine if leaching occurred, the sensor was incubated in pH 7.4 PBS buffer for 90 min, with measurements carried out every 5 min. The fluorescent intensities remained essentially constant (**Figure 7** (b)), indicating very little evidence of the fluorophores leaching from the thin polymer film at the end of the fibre.

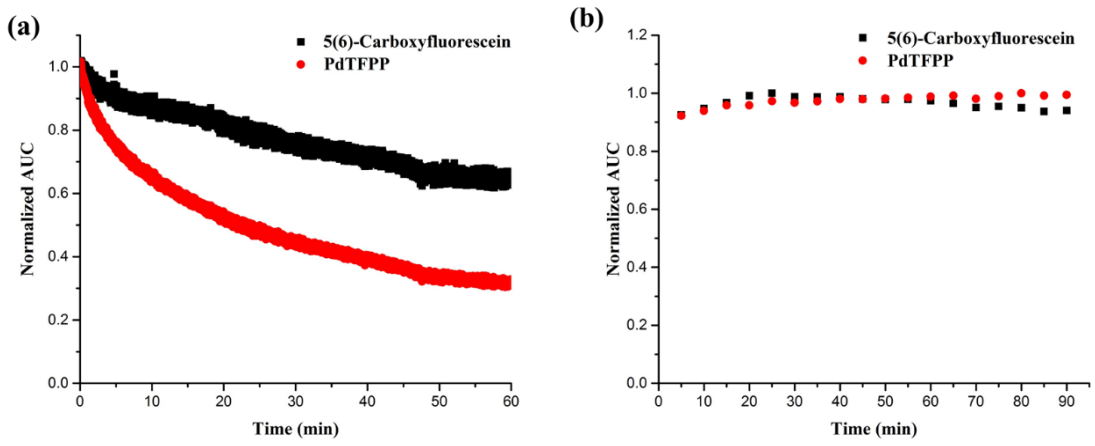


Figure 7. (a) Photobleaching and (b) leaching tests of the sensors in pH 7.4 phosphate buffer showed significant photo-bleaching (60 min continuous exposure to a laser excitation at 485 nm with a power of 1.0 μW) showing negligible leaching of either dye.

CONCLUSION

We have developed a thin-film polymer-based optical pH sensor based on ratiometric fluorescence measurement. The 5(6)-carboxyfluorescein/PdTFPP sensor had a good dynamic range and reproducibility responding rapidly to pH changes. The 39:1 molar ratio of 5(6)-

carboxyfluorescein/PdTFPP was observed to be optimal for use as a ratiometric pH sensor as a result of fluorescence energy transfer from 5(6)-carboxyfluorescein to PdTFPP. Ratios (see **Figure S4**) lower than 20:1 resulted in very strong PdTFPP emission, with little emission of 5(6)-carboxyfluorescein observed, while a ratio of 195:1 resulted in strong 5(6)-carboxyfluorescein emission that overwhelmed the intensity of the PdTFPP. To validate the capabilities of this fabricated optical fibre probe in biomedical applications, we used the probe to measure the pH of a sample of ovine lung tissue (see **Figure S5**). This showed the probe to be accurate, sensitive and rapidly responsive to pH and should provide the ability to measure subtle pH changes in biological systems. Importantly the hit polymer **PA101** was discovered using polymer microarray screening and this is the first report of polymer microarrays being used to identify hit polymers for the immobilization of pH sensors and will enable a much more robust approach to identify optimal coating polymers for a range of sensing applications. The chemical composition of the hit polymer PA101 is largely based on MMA, that will give a hydrophobic polymer that will help trap the aromatic fluorophores as well as offering stability in water. At the same time PA101 contains a monomer with a tertiary amine group that will be charged at physiological pH thus offering the means to both interact with/bind 5(6)-carboxyfluorescein as well as promoting proton transfer through the polymer to the sensor.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXX.

PA101 synthesis and characterization; table of fold increase in fluorescence for the polymer microarray screening; figures showing the epi-fluorescence system, emission spectra of 5(6)-carboxyfluorescein, PdTFPP and the optical fibre 5(6)-carboxyfluorescein/PdTFPP sensors;

experiment for pH measurement on the ovine tissue sample; matlab script for fluorescent sensor distribution analysis on the end of the fibre (PDF)

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Notes

The authors declare no competing financial interest.

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