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Localised hydrogen peroxide sensing for reproductive health

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ABSTRACT

The production of reactive oxygen species (ROS) is known to affect the developmental competence of embryos. Hydrogen peroxide (H_2O_2) an important reactive oxygen species, is also known to causes DNA damage and defective sperm function. Current techniques require incubating a developing embryo with an organic fluorophore which is potentially hazardous for the embryo. What we need is a localised ROS sensor which does not require fluorophores in solution and hence will allow continuous monitoring of H_2O_2 production without adversely affect the development of the embryo. Here we report studies on such a fibre-based sensor for the detection of H_2O_2 that uses a surface-bound aryl boronate fluorophore carboxyperoxyfluor-1(CPF1). Optical fibres present a unique platform due to desirable characteristics as dip sensors in biological solutions. Attempts to functionalise the fibre tips using polyelectrolyte layers and (3-aminopropyl)triethoxysilane (APTES) coatings resulted in a limited signal and poor fluorescent response to H_2O_2 due to a low tip surface density of the fluorophore. To increase the surface density, CPF1 was integrated into a polymer matrix formed on the fibre tip by a UV-catalysed polymerisation process of acrylamide onto a methacrylate silane layer. The polyacrylamide containing CPF1 gave a much higher surface density than previous surface attachment methods and the sensor was found to effectively detect H_2O_2. Using this method, biologically relevant concentrations of H_2O_2 were detected, enabling remote sensing studies into ROS releases from embryos throughout early development.

INTRODUCTION

Reactive oxygen species (ROS) produced in reproductive cells are known to cause DNA damage, resulting in defective sperm function[1-3] and compromised embryo development.[4] Hydrogen peroxide (H_2O_2) is an important ROS in reproductive health, and its detection is vital for monitoring embryo health and the diagnosis of male infertility. H_2O_2 is typically detected in cells using a compound (fluorophore) that fluoresces on its reaction with ROS.[5, 6] However, the use of such probes during *in vitro* fertilisation (IVF) applications poses significant scientific and ethical problems. The effect of these compounds on embryo development is unknown, and as such direct contact is not advisable. Hence, non-invasive and non-toxic diagnostics are highly sought after by clinical laboratories. One possible alternative to solution-based measurements is to chemically attach the fluorophore to a surface to allow localised measurement without releasing the fluorophore.[7-9] Thus, by immobilising a H_2O_2 sensitive fluorophore peroxyfluor-1 (PF1) to glass surfaces, detection of H_2O_2 can be performed without potential detrimental effects on the embryo.

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Aryl boronates such as PF1 are known to fluoresce on reaction with H_2O_2 [10-12] and we have also recently demonstrated that PF1 and other variants are particularly effective probes for detection of H_2O_2 in human spermatozoa [13] and bovine oocytes [14]. This paper reports the attachment of synthetic carboxy peroxyfluor-1 (CPF1) and N-hydroxysuccinimide CPF1 (NHS-CPF1) to confocal microscope slides and optical fibres for remote sensing of H_2O_2. Both direct covalent attachment of the fluorophore to the glass surface, as well as the attachment of a polymer layer with the fluorophore embedded are demonstrated below.

METHODS

Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. CPF1 and PF1-CONHS were synthesised by known procedures.[11, 13] Bovine IVF medium was prepared using VitroFert from IVF Vet Solutions (Adelaide, Australia); 4 mg/ml fatty acid free BSA (ICPBio Ltd; Auckland, New Zealand); 10 IU/ml heparin, 25 μM penicillamine, 12.5 μM hypotaurine and 1.25 μM epinephrine. Bis(acrylamide) was purchased from Polysciences (Warrington, PA). HPLC grade toluene was purchased from Southern Cross Science (Adelaide, AUS), and dried using a Puresolv solvent purifier from Innovative Technology (Amesbury, MA). HPLC grade methanol and acetonitrile was purchased from Scharlau. 200 μm core diameter multimode fibre was purchased from Thorlabs (USA). Milli-Q water was purified by a Millipore purification system (Billerica, MA). Glass bottom, confocal microscope dishes were purchased from Cell E&G (Houston TX).

Fluorescence Characterisation

The response of CPF1 to H_2O_2 was measured using a Biotek Synergy H4 plate reader. CPF1 was made up to a final concentration of 10 μM in 20 mM HEPES buffer at pH 7.4. H_2O_2 was added to give final concentrations of 0, 10, 25, 50, 75 and 100 μM. Absorption and fluorescence spectra were measured after 40 min of incubation at 37°C.

Surface Attachment Protocols

Preparation of Confocal Microscope Dishes

Confocal microscope dishes were washed with Milli-Q water before being sonicated in HPLC grade methanol for 20 min and dried under vacuum for 1 h. Stock solutions of polyallylamine hydrochloride and polyacrylic acid were made up to 1 mg/mL in a 1 M sodium chloride solution. 1 mL of polyallylamine hydrochloride stock was carefully pipetted onto the exposed glass surface of the cleaned confocal dish and left for 15 min. The polyallylamine hydrochloride solution was decanted, and the surface carefully rinsed 4x with 2.5 mL of Milli-Q water. 1 mL of polyacrylic acid stock was then pipetted onto the glass surface and left for 15 min. After decanting, the glass was rinsed with a further 4x 2.5 mL of Milli-Q water. A further layer of polyallylamine hydrochloride was deposited and rinsed as previously described. 1 mL of an aqueous solution of EDC•HCl (1 mM), NHS (1 mM) and CPF1 (10 μM) was pipetted onto the glass and left for 1 h. The excess was decanted and the dish washed with 4x 2.5 mL of Milli-Q water and placed under vacuum for 12 h to remove excess water.

5 μL droplets of Bovine IVF medium with increasing concentrations of H_2O_2 (0-500 μM) were added to the plates and covered with paraffin oil (Merck; Darmstadt, Germany) to eliminate evaporation, then incubated at 38.5 °C for 1 h. The fluorescence of individual media drops on the plates was captured using an Olympus (Tokyo) Fluoview FV10i (ex = 470 nm and em = 500-600 nm), before being imaged on the Amersham Typhoon imager as per below.

The fluorescence generated on the glass slides before and on reaction with H_2O_2 was measured using a Typhoon TM 8600 variable mode imager from Amersham Bioscience. Excitation was performed using a 488 nm laser,

and the emission measured through a 520 nm filter with a 40 nm bandwidth. Sensitivity was set to 100 μm per pixel, and photomultiplier tube voltage was 500 V. Average fluorescence intensity per mm^2 was collected from 100 data points and the error was calculated to give the standard error of the mean (SE).

Polyelectrolyte Deposition on Optical Fibre Tips

Stock solutions of polyallylamine hydrochloride, polyacrylic acid (1 mg/mL in a 1 M sodium chloride) and a 100 μM CPF1-NHS solution with 1 mg/mL polyallylamine hydrochloride in 1M sodium chloride were prepared. The freshly cleaved tip of a length of multi-mode fibre (200μm) was immersed in the polyallylamine hydrochloride solution for 5 min, rinsed in Milli-Q water and then re-immersed in polyacrylic acid for 5 min. This process was repeated 8 times to give alternating layers of polyallylamine hydrochloride and polyacrylic acid. The fibre tip was finally immersed in the polyallylamine hydrochloride solution containing CPF1-NHS for 5 min and then washed and immersed in polyacrylic acid for 5 min. This process was repeated to give 3 layers of polyallylamine hydrochloride containing CPF1-NHS on the fibre tip.

Silane Functionalisation on Optical Fibre Tips

Multi-mode fibre (200 μm diameter) was cut to length and the end face was cleaved in order to provide a clean surface. Under a dry nitrogen atmosphere, the fibre tips were washed with ethanol followed by dry toluene for 30 min. The fibre tips were then functionalised by placing them in a 5% mixture of (3-aminopropyl)triethoxysilane (APTES) in toluene before a further wash with dry toluene. The fibre was removed from nitrogenous atmosphere and washed with HPLC grade acetonitrile. The tip was immersed in a mixture of EDC•HCl (1 mM), NHS (1 mM) and CPF1 (100 μM) in HPLC grade acetonitrile. The fibre tip was then removed, washed with further acetonitrile and Milli-Q water for use on an optical fibre setup.

Polyacrylamide Photopolymerisation on Optical Fibre Tips

A solution of 3-(trimethoxysilyl)propyl methacrylate in pH 3.5 adjusted Milli-Q water was mixed and sonicated until clear. Multi-mode fibre (200 μm diameter) was cut to length and the end face was cleaved in order to provide a clean surface, then immersed in the methacrylate solution for 1 h. The fibre tip was then dried under N_2, rinsed with Milli-Q water and dried under N_2 again. The distal end of the fibre was then coupled into the fibre setup shown in Figure 1 below. A monomeric stock solution comprising of 3% bisacrylamide, 27% acrylamide and 70% pH 6.5 phosphate buffer solution was dissolved under sonication. CPF1-NHS (0.2 mg) was dissolved in the monomeric solution (400 μL) with triethylamine (10 μL) and 200 μL of this was pipetted into a small Eppendorf tube. The functionalised fibre tip was immediately immersed in this solution, and irradiated under UV light for 2s to form a polymeric coating on the fibre tip.

Fibre Setup

A schematic of the optical setup is shown in Figure 1, for both photopolymerisation of the polymer using the 405 nm source, and optical measurements using the 473 nm source.

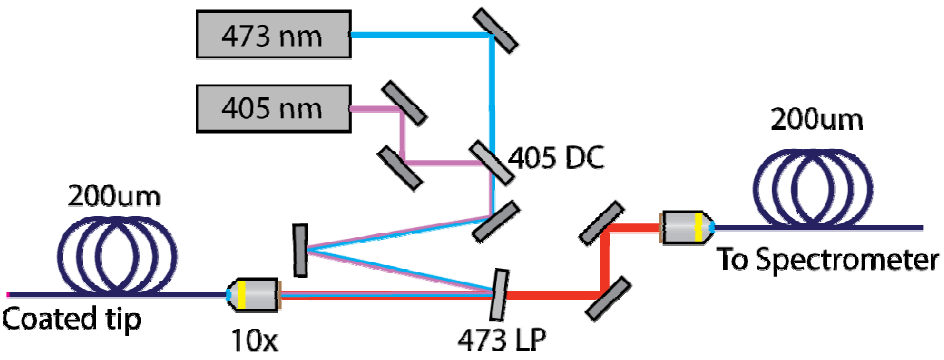


Figure 1 - Experimental configuration for photopolymerisation, and optical measurements

For excitation of the fluorophore for both direct attached and polymer embedded methods, the 473 nm laser (Toptica iBeam Smart) was coupled into the distal end of the probe fibre, with the 405 nm arm blocked. The coupled laser light then excites the fluorophore-doped probe tip, and a portion of this fluorescent light is then captured into a back-propagating mode in the fibre. This then passes through a 473 nm long-pass filter (Semrock EdgeBasic) to remove excess pump light, before being coupled into a spectrometer (Horiba iHR320) via a 200 µm optical fibre patch cable.

The same experimental configuration was used to induce photopolymerisation on the fibre tips, with the 473 nm path blocked, and a timed shutter used on the 405 nm source to control the deposition time of the polymer.

RESULTS AND DISCUSSION

Response of CPF1 to H_2O_2 on Glass Slide

CPF1 was incubated in media with differing concentrations of H_2O_2 and the fluorescent response measured by a fluorescence plate reader (Figure 2) in order to define the response of CPF1 to H_2O. A significant fluorescent response was measured after 40 min exposure of CPF1 to 10 µM of H_2O_2. This demonstrates a high sensitivity of detection of H_2O_2 at biologically relevant concentrations. Furthermore, increasing the concentrations of H_2O_2 gave an increased fluorescent response from CPF1. This is in good agreement with our previous studies which showed this class of probe to be effective in the detection of H_2O_2 in human sperm cells [13] and for indicating oxidative stress in bovine embryos.[14]

Glass confocal microscope dishes were functionalised with CPF1 to compare the relative fluorescent responses of CPF1 to H_2O_2 on a glass surface and in solution. CPF1 was covalently linked to free amines of a poly(allylamine hydrochloride) which had been deposited on glass slides with poly(acrylic acid) in a layer-by-layer deposition technique (see Methods). These functionalised slides were then immersed in a H_2O_2 solution to oxidise the probe and increase fluorescence. The typhoon imager was then used to measure the resulting surface fluorescence, see Figure 3. This data confirms successful attachment of CPF1 to the polyelectrolyte layers due to the large increase from the control sample with polyelectrolyte only. Moreover, a significant increase in fluorescence is shown by the samples treated with H_2O_2, indicating that there is a response of the surface-bound CPF1 to H_2O_2 as in solution.

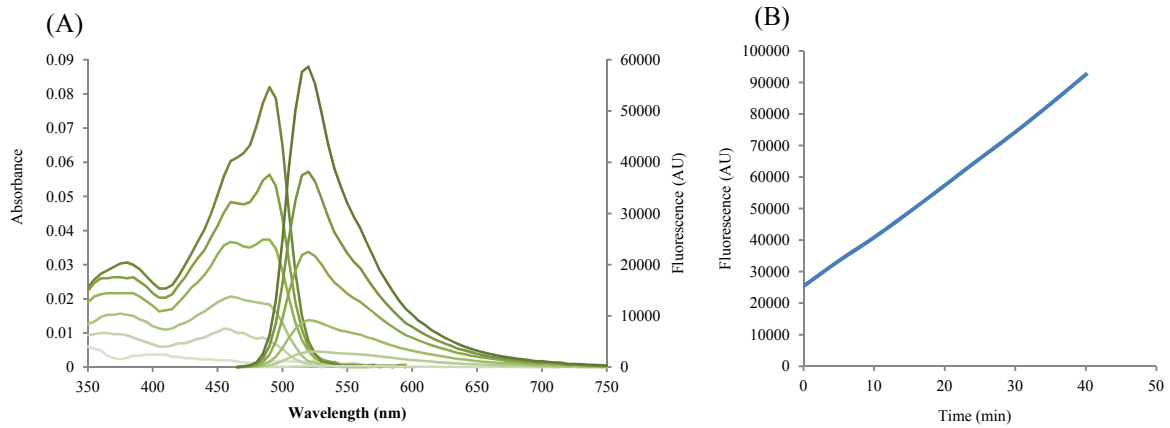


Figure 2. (A) Absorption and emission spectra of CPF1 in 20 mM HEPES solution when treated with 0, 10, 25, 50, 75 and 100 µM H_2O_2. (B) Fluorescence over 40 min of CPF1 treated with 100 µM H_2O_2. Excitation was at 450 nm and emission at 520 nm with a band pass of 20 nm.

The sensitivity of surface-bound CPF1 to H_2O_2 was next defined. Dishes were again functionalised with CPF1 and 5 µL drops of bovine IVF media containing 0, 10, 50, 100 or 500 µM of H_2O_2, were added and overlaid with paraffin oil. These dishes were imaged using a typhoon imager (Figure 4A) and also a confocal microscope (Figure 4B-E). The results clearly show that higher concentrations of H_2O_2 lead to a greater fluorescent response from the surface bound CPF1. This indicates that, while bound to a surface, CPF1 is effective for the detection of H_2O_2 in IVF media.

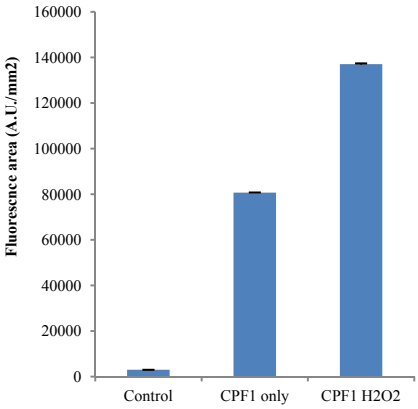


Figure 3. Characterisation of glass slide functionalised with CPF1 on polyelectrolytes. The above data is an analysis of the fluorescence measured by typhoon imaging.

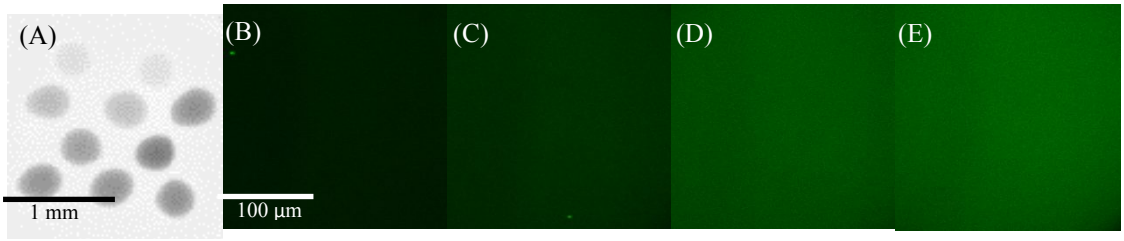
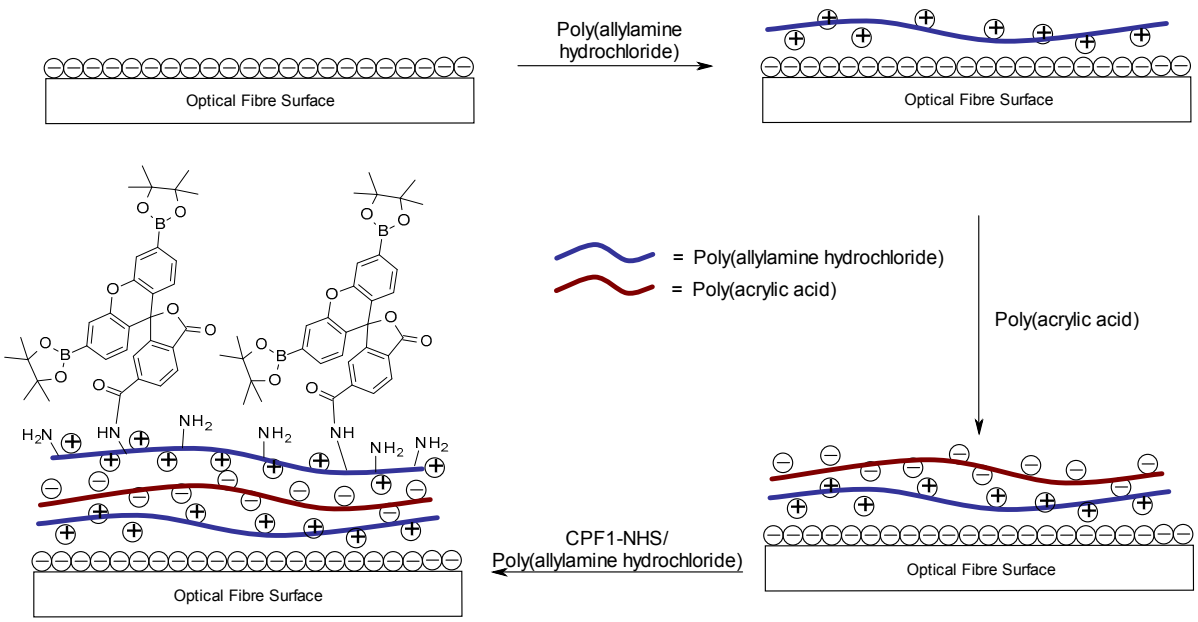


Figure 4. A glass slide functionalised with CPF1 has several droplets with differing concentrations of H_2O_2. (A)Typhoon image with concentrations increasing from left to right, top to bottom. Confocal microscope images corresponding to these droplets show increasingly bright fluorescence for increased H_2O_2: (B) 0µM (C) 10µM (D) 50µM (E) 100µM**.**

Attachment Methods of CPF1 to Fibre Tip

Having established that CPF1 can detect H_2O_2 on a glass surface, three polymer and silane functionalisation methods were trialled for immobilising CPF1 to a fibre tip. In this case CPF1-NHS was used in place of CPF1, an activated ester which directly attaches to amines on the surface. Polyelectrolyte deposition was first investigated (see Scheme 1), as per attaching CPF1 to confocal glass slides. Positively charged polymer polyallylamine hydrochloride and negatively charged polyacrylic acid were immobilised to the surface of the cleaved fibre tip by electrostatic attraction. 15 layers were deposited on fibre tip, a larger number compared with the 3 layers on confocal slides, to ensure a dense surface coverage for increased fluorophore signal. These polymers are not covalently bound to the surface, but are held in place by electrostatic attraction, that are compatible with a biological pH.[15]

The fibres functionalised with CPF1 were then coupled into a 473 nm light source for detection of any increase in fluorescence on exposure to H_2O_2. The tip of the fibre was dipped into 1 mM H_2O_2 and the fluorescence monitored over 20 min, with the results shown in Figure 5. No significant increase in fluorescence was apparent over the 20 min, indicating that the majority of the observed signal is background fluorescence on the glass fibre, and thus a low surface density of the CPF1 on the fibre tip. By comparison, the use of polyelectrolytes on glass slides (as described above) was effective at detecting an increase in H_2O_2. This difference could be due to the higher efficiency of the confocal microscope and typhoon imager for collecting fluorescence signal, as well as the lower power setting used in fibre to minimise photobleaching. It is clear that while polyelectrolyte deposition affords an acceptable level of CPF1 on the glass confocal microscope slides, there is insufficient CPF1 deposited on a fibre tip for H_2O_2 sensing.



Scheme 1. Representative diagram of the deposition of poly electrolyte layers polyallylamine hydrochloride and polyacrylic acid to a glass surface. The process is repeated to build up multiple layers of alternating charge, with functionalisation of CPF1 to the top layers. The final three deposited layers of polyallylamine hydrochloride contained CPF1-NHS

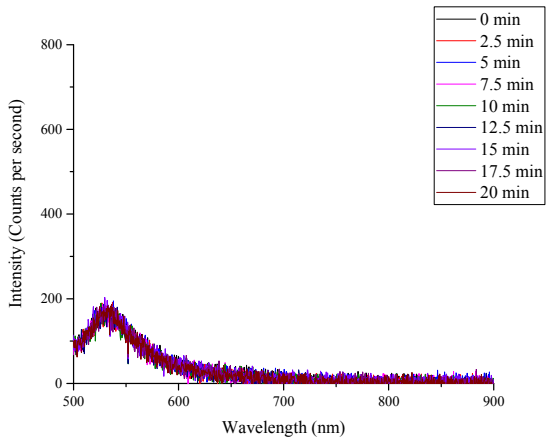
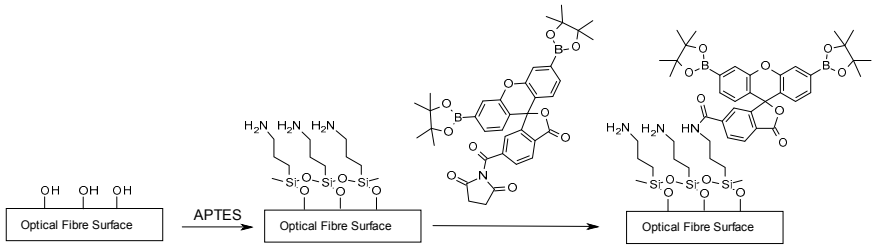


Figure 5. Poly electrolyte coating with CPF1 attached, in 1 mM H_2O_2 for 20 min. The lack of fluorescent increase indicates the poor signal on the fibre tip.

The second method involved depositing an amine-functional silane layer on the fibre tip (Scheme 2). This was achieved by dipping the fibre tip into a mixture of APTES under anhydrous conditions to give even surface coverage, before attaching CPF1-NHS. The fibre was then coupled to a 473 nm laser and the fluorescence measured over 20 min when dipped into a 1 mM solution of H_2O_2 in bovine IVF media. Figure 6 shows that the fluorescence increases when CPF1 bound to an APTES monolayer on the fibre tip reacts with H_2O_2. A slight increase in the fluorescence maximum at 520 nm is apparent, but this increase is insufficient for the detection of *in vitro* concentrations and is comparatively poor relative to CPF1 in solution (Figure 2).



Scheme 2. Surface functionalisation of CPF1-NHS with APTES. Deposition of APTES to the fibre surface is performed in anhydrous conditions to avoid polymerisation and create an even monolayer.

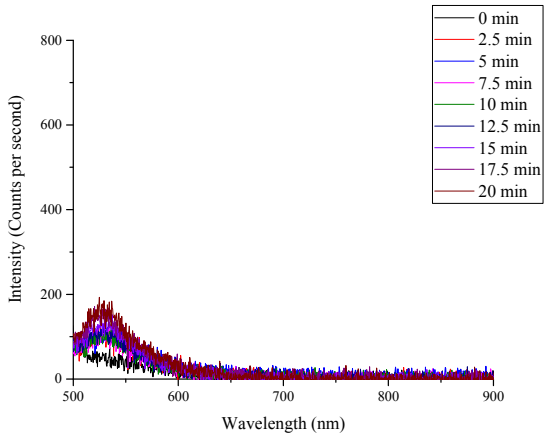
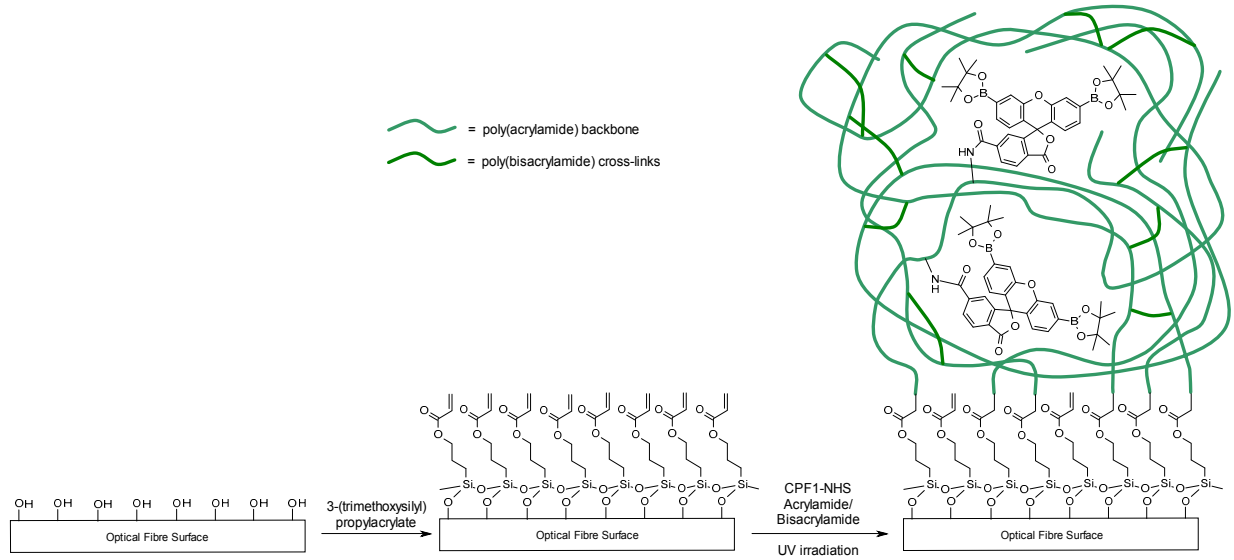


Figure 6. APTES-coated fibre tip gives poor increase in fluorescence to 1 mM H_2O_2.

The third method of functionalisation involved forming a poly(acrylamide) matrix containing CPF1 on the glass surface (Scheme 3). A silane monolayer was first formed on the fibre tip using an aqueous solution of 3-(trimethoxysilyl)propyl acrylate. The fibre tip was then dipped into a solution of acrylamide/bisacrylamide while 405 nm light was coupled into the fibre catalysing polymerisation to the silane acrylate on the surface. The thus formed cross-linked poly(acrylamide) effectively traps the fluorophore in the polymer matrix, while allowing small molecules such as H_2O_2 to diffuse through it. The fibre tip was then washed, and 473 nm light was coupled into the fibre to measure the fluorescence of CPF1.

Figure 7 shows the increase of fluorescence over 20 min when the coated fibre tip was immersed in 1mM H_2O_2. A near linear increase can be seen in the fluorescence maximum over 20 min (Figure 7B), indicating a kinetic response of CPF1 to H_2O_2 similar to the initial solution-based studies (Figure 2B). It is clear that the poly(acrylamide) coating method gives a higher surface density of CPF1 on the fibre tip compared to the polyelectrolyte (Figure 5) and APTES (Figure 6) functionalisation methods. Furthermore, these fibres effectively sense H_2O_2 and have potential for use as a dip-sensor for H_2O_2 near embryos.



Scheme 3. Formation of a poly(acrylamide) matrix with covalently linked CPF1. A silane layer is deposited with propylacrylate functionality. The polymer is then formed on the tip by immersing the fibre tip in an aqueous solution of CPF1-NHS in acrylamide/bisacrylamide and irradiating through the fibre with a 405 nm laser source.

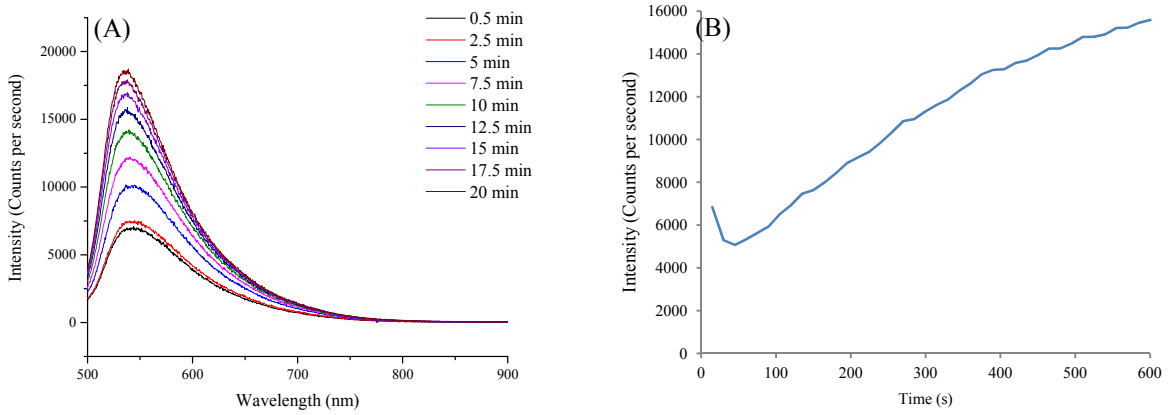


Figure 7. The response of CPF1 in poly(acrylamide) to 1 mM H_2O_2 (ex. 473 nm laser): (A) Overlaid spectra over 20 min of exposure to H_2O_2. (B) Intensity change over time at emission peak 520nm. The initial drop in fluorescence at 0s represents the fibre tip being dipped into solution; the fluorescence is quenched slightly in water compared with air.

CONCLUSION

CPF1 was demonstrated to effectively detect H_2O_2 when bound to a surface by either polyelectrolyte deposition on glass slides, or in poly(acrylamide) on an optical fibre tip. This fibre-based sensor shows promising results for remote H_2O_2 sensing, as the poly(acrylamide) coating method gave a high surface density of fluorophore. Use of this fibre coating method is now being investigated for non-invasive detection of oxidative stress near embryos.

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