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Microstructured Optical Fiber-based Biosensors: Reversible and Nanoliter-Scale Measurement of Zinc Ions.

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ABSTRACT

Sensing platforms for the rapid and efficient detection of metal ions would have applications in disease diagnosis and study, as well as environmental sensing. Here we report the first microstructured optical fiber-based biosensor for the reversible and nanoliter-scale measurement

of metal ions. Specifically, a photoswitchable spiropyran Zn^{2+} sensor is incorporated within the microenvironment of a liposome attached to microstructured optical fibers (exposed-core and suspended-core microstructured optical fibers). Both fiber-based platforms retains high selectivity of ion binding associated with a small molecule sensor, while also allowing nanoliter volume sampling and on/off switching. We have demonstrated that multiple measurements can be made on a single sample without the need to change the sensor. The ability of the new sensing platform to sense Zn^{2+} in pleural lavage and nasopharynx of mice was compared to that of established ion sensing methodologies such as inductively coupled plasma mass spectrometry (ICP-MS) and a commercially available fluorophore (Fluozin-3), where the optical fiber based sensor provides a significant advantage in that it allows the use of nL sampling when compared to ICP-MS (mL) and FluoZin-3 (μL). This work paves the way to a generic approach for developing surface-based ion sensors using a range of sensor molecules, which can be attached to a surface without the need for its chemical modification and presents an opportunity for the development of new and highly specific ion sensors for real time sensing applications.

KEY WORDS: Biosensor, Microstructured Optical fiber, Photoswitch, Liposome, Zinc, Nanoscale

INTRODUCTION

The ability to detect the on/off binding of a metal ion to a complementary surface-bound receptor provides a basis for real-time sensing applications in fields such as environmental monitoring and in clinical diagnostics. Small-molecule 'on/off' sensors for metal ions, such as those based on rhodamine[1-3] or other fluorophores,[4-5] can exhibit high selectivity for a given ion

and as a result have found wide use in this context. However, chemically modifying these small molecules to allow surface attachment, while retaining efficient sensing capability, can be problematic from both a synthesis and an operational viewpoint as the immobilized molecule may lose functionality.[6] Sensing can also be achieved using a larger biological receptor such as a protein or nucleic acid capable of binding an ion of interest.[7] While these systems allow for better surface attachment through standard coupling with a component amino acid or another biomolecule within, these biological receptors are typically only weakly selective for a given ion. Although this can be improved somewhat by genetic modification, this approach is time consuming and lacks broad generality.[7-8] A generic and biocompatible approach to attach highly selective small molecule sensors to surfaces, without the need for chemical modification, would offer significant advantages over these methods.

Here we report such an approach where the phospholipids and a photochromic sensor molecule are assembled to form a novel liposome-based sensing material and attached this to the surfaces of microstructured optical fibers. This new sensing platform retains high selectivity of ion binding associated with a small molecule sensor, while also allowing sampling of small volumes and an opportunity for on/off switching. Liposomes were chosen because they can be readily made from a range of natural lipids and are nontoxic and biodegradable.[9] Detection of Zn^{2+} was incorporated into the design of this new sensing platform because of the essential role of Zn^{2+} in a range of cellular processes[10] such as antioxidant enzyme activity,[11] DNA structural integrity, oocytes maturation and fertilization[12] while disruption of its homeostasis is associated with numerous disease states including Alzheimer's,[13] diabetes[14] and cancer.

A photoswitchable spiropyran-based ion chelator (**SP2**) was chosen for embedding within the surface of the microstructured optical fiber-bound liposome as shown in Figure 1. **SP2** contains

an aryl carboxylate group for improved aqueous solubility and the bis(2-pyridylmethyl)amine functionality was chosen as the ionophore in this work as it is known to selectively form a complex with Zn^{2+} over other biologically relevant ions in solutions (Supporting information Figure S1).[15] The photoswitching of spiropyran on surfaces[16] and semi-condensed phase[17-19] are well-characterized and in this context, exposure of this compound to Zn^{2+} (by sampling through the optical fiber) gives rise to a ring-opened and fluorescent merocyanine isomer that is stabilized by specific binding through the appended phenoxide and bis-pyridyl functionalities.[15] This switching can also be induced on illumination with UV through the fiber. Irradiation with white light regenerates the ring closed spiropyran isomer with release of Zn^{2+}, resulting in a switchable sensing platform. In all cases fluorescence is both induced and measured through the microstructured optical fibers, which is exposed to the 532 nm excitation light for 10 ms 10 times. The long interaction length between the evanescent tails of the guided light and the material attached to the surface of the optical fiber maximizes the fluorescence signal to give improved sensitivity of detection relative to more traditional spectroscopic and fluorescence-based fiber sensors.[20] Two different types of microstructured optical fibers were used in these experiments. The first is an exposed-core microstructured optical fibers (ECF) that has a suspended micron-scale core partially exposed to the external environment[21-22] (Figure 2, left), which allows consistent washing, drying and refilling of the biosensor during its use. The second type of fiber has air holes within its cross section and is known as a suspended-core microstructured optical fibers (SCF),[23] see Figure 2 right. These air holes confine light to the solid core and allow it to be guided along the length of the fiber. The holes also act as micro sample chamber to allow nanoscale sampling[20], a real advance in biological sensing.

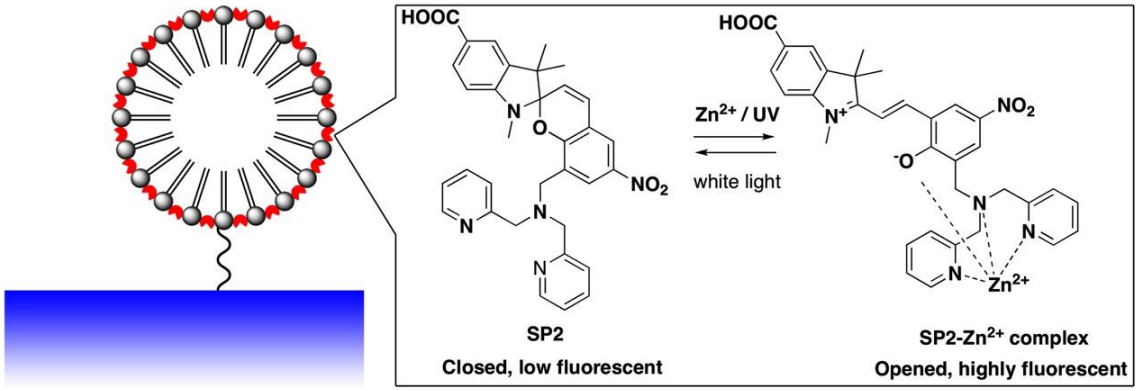

Figure 1. Schematic of the optical fiber-based sensing architecture**,** where a photoswitchable spiropyran (**SP2**) (red) is embedded within a liposome (grey). This structure (**SP2-liposome)** is covalently attached to the surface of an ECF or SCF (blue). The insert shows the isomeric structures of spiropyran **SP2** (closed, non-fluorescent spiropyran isomer), and the metal-induced ring-opened Zn^{2+} complex (opened, fluorescent merocyanine isomer). The spiropyran-Zn^{2+} sensing mechanism depicted here was modeled by Rivera-Fuentes et al[24] on a similar analog using density functional theory calculations.[24] The ring-opened isomer is induced by binding to Zn^{2+} or by exposure to UV light (λ_{em} = 320 – 350 nm), while the ring-closed isomer is induced by exposure to white light (broad spectrum).

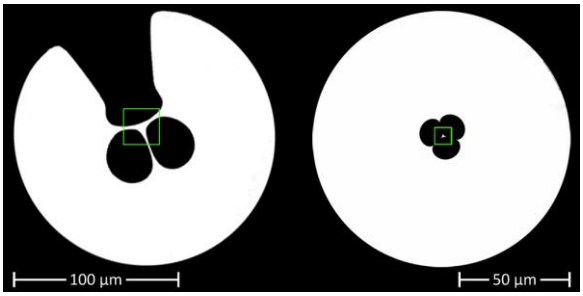

Figure 2. Scanning electron microscopy images of the microstructured optical fibers used in this work. (Left) the silica ECF and (Right) the silica SCF, with effective core diameters of 7.5 μm and 1.5 μm respectively. The green box highlights the core of each fiber.

RESULTS AND DISCUSSION

Optimum conditions for the generation of a functional and stable integrated spiropyran-liposome system (**SP2-liposome**) were initially investigated, where liposome-based materials are known to be unstable when dehydrated. Liposomal stability is especially important for reusable sensors of the type developed here since measurement would require cycles of washing and air-drying between readings. Dehydration of the liposome could also occur during long-term storage of the sensor. Such factors have limited the development of liposome-based sensors to date.[9, 25] With this in mind, a solution of **SP2** in DMSO was mixed with a solution of total *Escherichia coli* lipids extracts in buffer containing various amounts of maltose or trehalose (0%, 5%, 10% or 20%, weight/vol) and 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS) at pH 7.2. The formation of hydrogen bonds between the sugar and surrounding polar residues of liposomes was expected to help prevent collapse and loss of function under the dehydrating conditions.

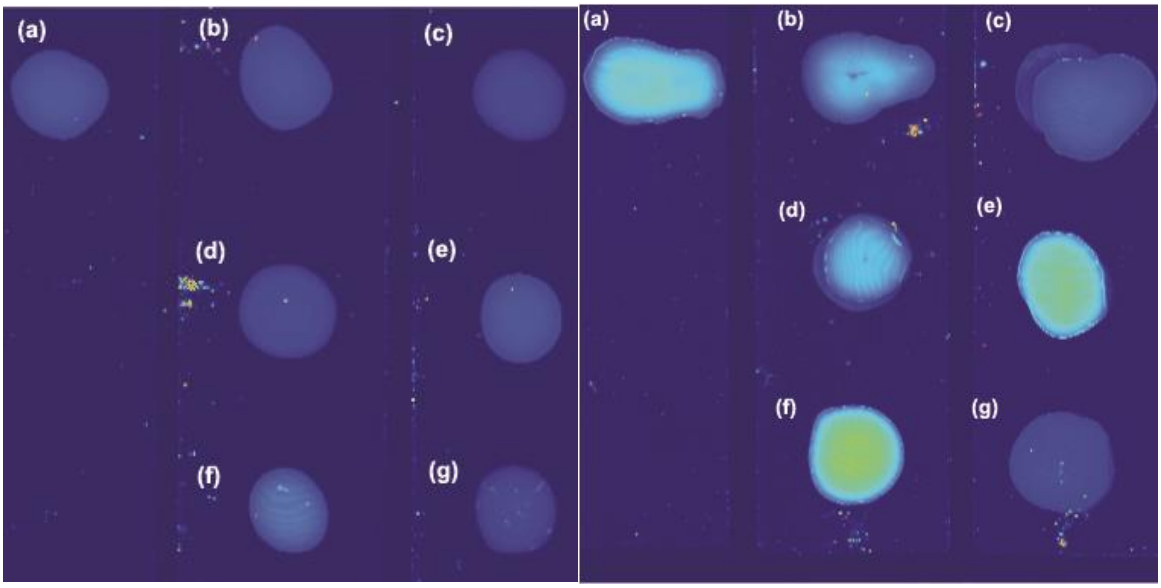

Figure 3. Initial studies were carried out on readily available silicate glass slides as a simple model for the microstructured optical fibers (F300 silica glass), where both are silicate glasses with wettable (hydrophilic) surfaces. Images were taken using a Typhoon Imager (λ_{Ex} = 532 nm, λ_{Ex} ~ 640 nm). On each slide: 5 µL droplets constituted with (a) 10 % maltose (b) 20 % trehalose (c) 5 % trehalose (d) 10 % trehalose (e) 5 % maltose (f) 20 % maltose (g) 0 % disaccharide. The glass slide on the left and right represent the droplets before and after the addition of 100 µM Zn respectively.

Samples of the above solutions were then placed on a glass microscope slide, initial fluorescence was measured by irradiating with the 532 nm laser using a Typhoon imager, and aqueous zinc chloride (100 µM) was added to each, the droplets were again irradiated (532 nm), and the resulting fluorescence of the complexed ring opened merocynine isomer measured, see Figure 3 and supporting information for detail. Each droplet was then irradiated with white light to expel Zn^{2+} ions and regenerate the passive spiropyran (non-fluorescent) isomer. The samples were then left to air dry for 18 h, rehydrated with further aqueous zinc chloride, and a subsequent off/on cycle was performed as above.

Samples prepared with 20 % of maltose and 10 % and 20 % of trehalose precipitated upon addition of Zn^{2+} and as such these conditions were deemed unsuitable for subsequent experiments. Importantly, the results shown in Figure 4 demonstrate that **SP2-liposomes** constituted with maltose 5% and 10% remained functional and underwent a second round of photoswitching after air-drying for 18 h and rehydration. In contrast, fluorescence emission of **SP2-liposome** prepared with 5 % trehalose did not return to intensity levels reflective of the ring-closed spiropyran when exposed to white light. The **SP2-liposome** samples lacking disaccharide were unable to photoswitch and functionality was not restored after rehydration, i.e. no modulation of fluorescence emission was observed (Figure 4a and b, red). Based on these results samples for subsequent experiments were prepared using the minimum amount of maltose (5%) that provided protection against drying (**SP2-maltose-liposome**).

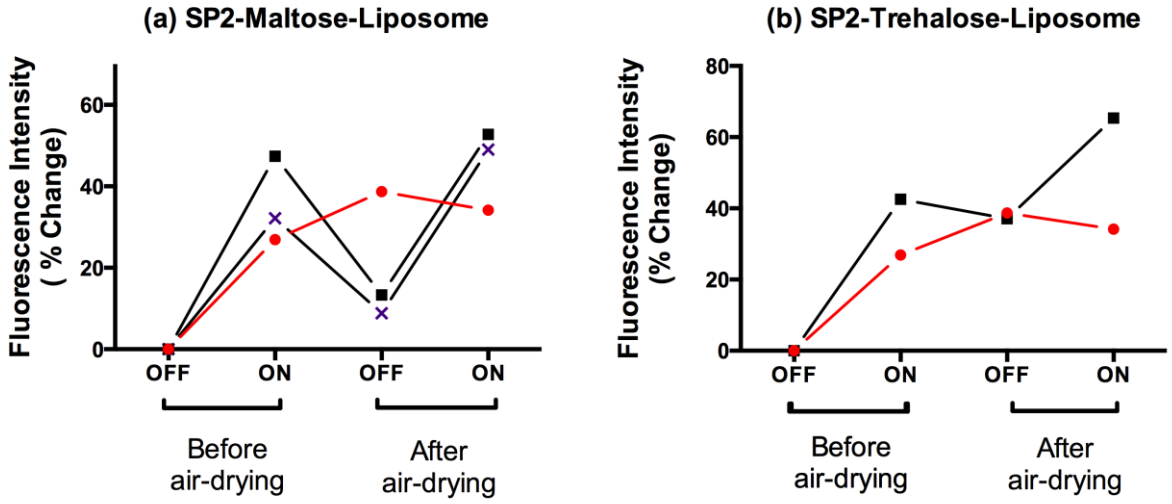

Figure 4. Photoswitching of the **SP2-liposome** on glass slide reconstituted with (•) 0 %, (■) 5 % and (**X**) 10 % maltose (Figure 4a) and (•) 0 %, (■) 5 % trehalose (Figure 4b) respectively. The experiments were performed on glass slides and resultant fluorescence was recorded using the Typhoon Imager (λ_{ex}= 532 nm). Each 'off-cycle' represents the fluorescence emission (λ_{em}

approximately = 640 nm) of the ring-closed spiropyran without Zn^{2+} chelated and after the droplets were irradiated with white light for 10 min. Each 'on-cycle' represents the resultant fluorescence emission (λ_{em} approximately = 640 nm) of ring-opened merocyanine isomer in the presence of 100 μM Zn^{2+} (**SP2**-Zn^{2+}). The fluorescence emission of the second 'off/on cycle' was obtained after the droplets had been air-dried for 18 h.

SP2-maltose-liposomes were next prepared with added succinyl PE lipid (as detailed in supporting information), where the component carboxylic acid would be expected to be exposed in the liposome structure to allow attachment to both exposed and suspended core microstructured fibers. Each fiber was precleaned and hydroxylated by immersion in nitric acid followed by piranha solution and then functionalized with alternating layers of poly(allylamine hydrochloride) (PAH), poly(acrylic acid) (PAA) and further PAH[26] for the exposed core fiber and APTES for the suspended core fiber.[27] The **SP2-maltose-liposomes** were then coupled onto to the surface-bound free amines using standard coupling conditions[27] (HATU/DIPEA, see supporting information) to give the functionalized exposed core and suspended core sensors.

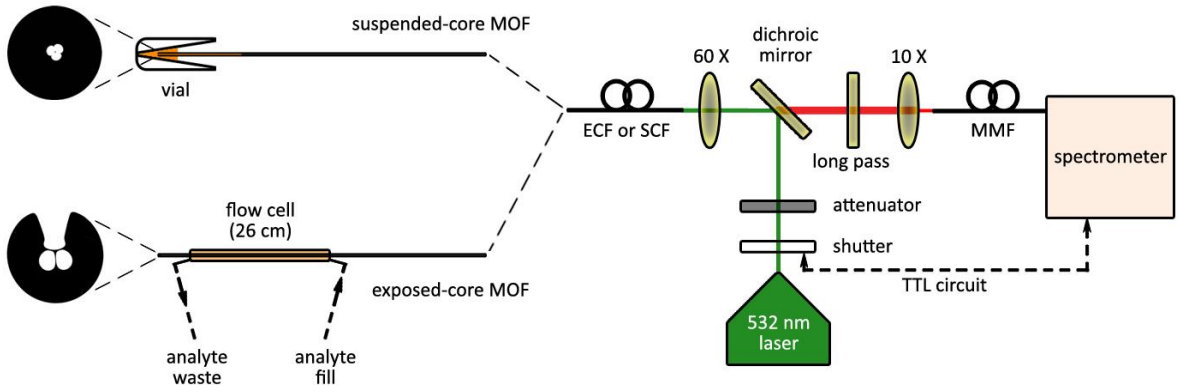

Figure 5. Setup used for fluorescence measurements using (top) suspended-core fiber and (bottom) exposed-core fibers.

The ability of the exposed core fiber-based sensor to bind and detect Zn^{2+} was studied using the optical set up depicted in Figure 5. A 5 mW laser excitation light source with a wavelength of 532 nm was coupled into the core of the functionalized fibers lengths using a 60X objective via a diachroic mirror to excite the sensing molecules and induce fluorescence at the surface of the core. The sensor was placed in a flow-cell filled with acetonitrile (Figure 5, exposed-core fiber, bottom) and then excited using a 532 nm laser (10 x 10ms pulses) and the resulting fluorescence measured. Figure 6a shows that the functionalized fiber (Fig. 6a, black) possessed significant background fluorescence (λ_{em} = 640 nm) relative to the unfunctionalized fiber (Figure 6a, green), as would be expected for spiropyran immobilized within the fiber's internal surface. Next, the flow cell was drained of acetonitrile; air-dried and refilled with a solution of Zn^{2+} in acetonitrile (10 μM). The fluorescence was again measured by excitation with the 532nm laser (10x10ms) and the resulting fluorescence emission intensity increased 2.5 fold (Figure 6a, red), which is consistent with formation of the highly fluorescent SP2-Zn^{2+} complex (Figure 1).[15] Next, the

sensor was irradiation with white light in order to release the bound Zn^{2+} and reform the ring-closed spiropyran isomer.[15] As expected, subsequent excitation of the sensor with the 532 nm laser showed the intensity of resultant fluorescence emission (Figure 6a, blue) returned to that of the sensor before Zn^{2+} was added (Figure 6a, black). After washing with acetonitrile to remove all Zn^{2+} the flow cell was again filled with solution of Zn^{2+} in acetonitrile (10 μM) and fluorescence measured as before in order to determine the reusability of the sensor. Again, fluorescence emission increased approximately 2.5 fold in the presence of Zn^{2+}. Irradiating the sensor with white-light and subsequently excitation with the 532 nm laser once again returned the fluorescence emission of the sensor to the level similar to that before the addition of Zn^{2+} (Figure 6a, black). The procedure was repeated for a third time and the results are summarized in Figure 6b. Importantly, attempted photoswitching of the microstructured optical fibers functionalized with **SP2-liposomes**, lacking disaccharide, failed to regenerate once the fibers had been drained of solution and left to air-dry.

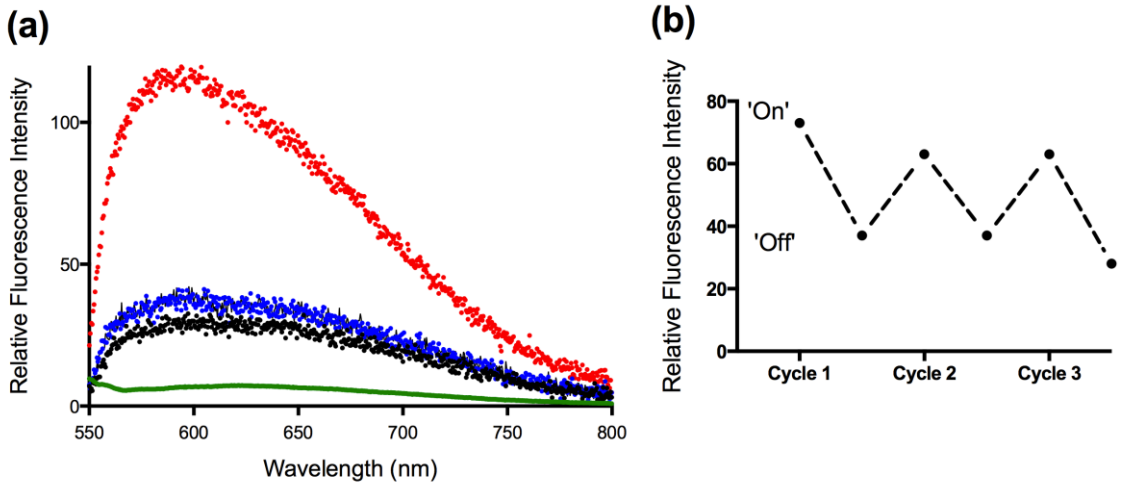

Figure 6. (a) Fluorescence emission spectra of exposed-core fiber functionalized with **SP2-maltose-liposome** after excitation with 532 nm laser; (green) Empty fiber, unfunctionalized, (black) exposed core based sensor in flow cell filled only with acetonitrile and no Zn^{2+}, (**red**) sensor filled 10 μM Zn^{2+} in acetonitrile and exposed to UV for 1 h, (blue) sensor, exposed to white light and rinsed with acetonitrile, to release Zn^{2+} from the sensor. (b) Photoswitching of functionalized exposed core-based sensor. 'Each cycle consist of filling the sensor with Zn^{2+} in acetonitrile (10 μM, 'On') followed by irradiating the sensor with white light for 30 min ('Off'). Fluorescence emission for each cycle was obtained by irradiation with the 532 nm laser.

Finally, the ability of the new sensing platform to sense Zn^{2+} in pleural lavage and nasopharynx of mice was compared to that of established ion sensing methodologies such as inductively coupled plasma mass spectrometry (ICP-MS) and solution fluorescence based (Fluozin-3) in order to validate its use in a biological setting. These two biological systems contain low endogenous Zn^{2+} concentrations, which results in poor signal-to-background ratios and a necessity to use of large sample volumes when using traditional ion-sensing techniques. Here we assessed the Zn^{2+} abundance in 8 separate murine samples using ICP-MS, Fluozin-3[28] and SCF sensor. Again the micron-sized air holes within the cross-section of the SCF allow control of the interactions between light guided within the fiber core and sensor located within the holes, while simultaneously acting as micro sample chambers. The experiments for ICP-MS and Fluozin-3 used established techniques[29] and are described in detail the supporting information. The functionalised suspended core based-sensor experiments were performed as follows; one end of the SCF was dipped into the biological sample and this mixture (20 nL) was drawn into the fiber air holes by capillary action (Figure 5, suspended core fiber, top). The

fluorescence was measured by excitation with a 532nm laser (10x10ms pulses) in each case and the resultant emission was recorded and shown in Figure 7. An approximate 3.5 and 4 fold increase in fluorescence intensities was observed for the nasopharyngeal (Figure 7, red) and pleural lavages (Figure 7, blue) samples, respectively. The sensing experiment was repeated using water in place of the biological samples as a negative control. No significant change in fluorescence was apparent in this case (Figure 7, black), which confirms that the earlier changes in fluorescence were due to the formation of the highly fluorescent SP2-Zn^{2+} species. An analysis of the pleural lavage across 8 murine samples showed that the optical fiber method was capable of detecting Zn^{2+} ions (uM levels, Figure 8a). Zn^{2+} concentrations were also quantified by Fluozin-3 (0.8-1.3 μM; Figure 8b) and ICP-MS (1.8-2.1 μM; Figure 8c). The results show that the narrow range of variation across the samples observed for the fiber-based approach (1.5-3.5 RFUs) correlates closely with the results for Fluozin-3. Minor differences in the absolute concentrations determined between ICP-MS and the fluorophore-based methods (Fluozin-3 and SCF-based sensor) were not unexpected as ICP-MS is based on a denaturing approach that releases ions from the biological material, while Fluozin-3 and the fiber-based approach use non-denaturing fluorophores that interact with the labile Zn^{2+} content in the biological samples. Importantly, the optical fiber based sensor provides a significant advantage in that it allows the use of nL sampling, which compares to ICP-MS (mL) and FluoZin-3 (μL).

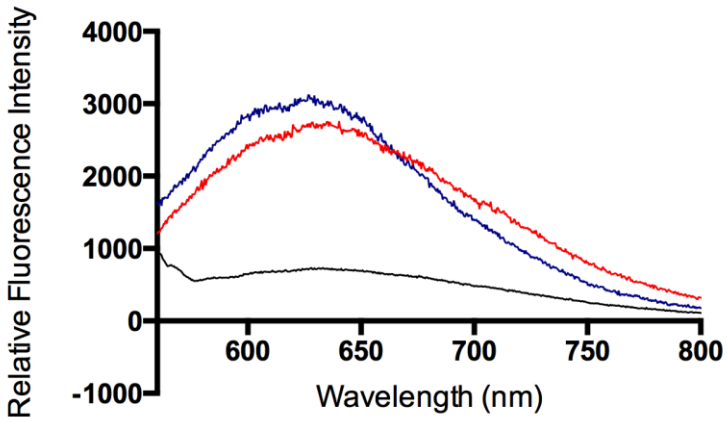

Figure 7. Representative fluorescence emission results from SCF sensor with biological samples. The emission data in the graph were obtained from (**black**) water only (**blue**) filled with solution from the pleural lavages (**red**) filled with solution from the nasopharyngeal wash. Emission data for all samples can be found in the supporting information section Figure S2.

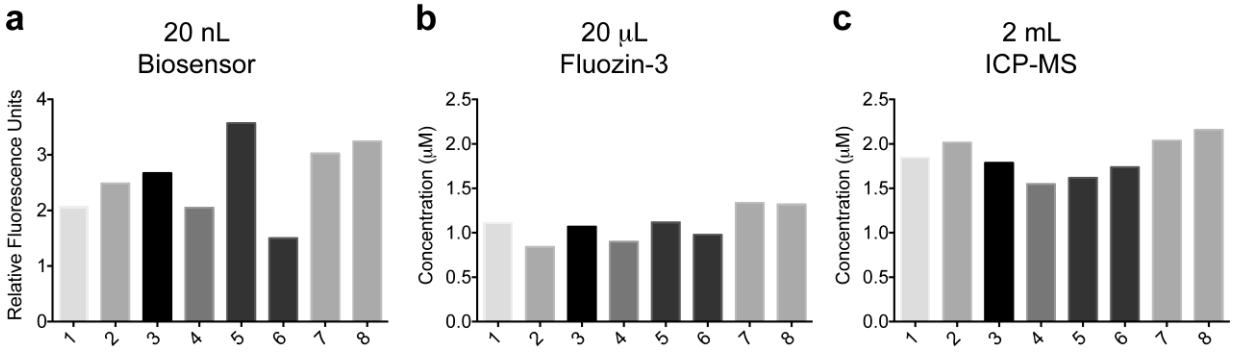

Figure 8. Analysis of Zn^{2+} in the pleural lavage samples obtained across 8 mice (numbered 1 to 8). (a) SCF direct detection of Zn^{2+} using nanolitre volumes (~20 nL) of sample. (b) Fluozin-3 detection of Zn^{2+} in biological sample using microliter volumes with added fluorophore (~20 µL). (c) ICP-MS determination of Zn^{2+} using 2 ml of processed sample

CONCLUSION

Metal ions are ubiquitous in the environment and biology and as such there is a real need to develop new sensing platforms for their rapid and efficient detection. Such sensors would have applications in disease diagnosis and study, as well as environmental sensing. Here we report the first instance where phospholipids and a photochromic sensor molecule are assembled to form a novel liposome-based sensing material. This was then coupled to microstructured optical fibers to create nanoscale biosensors that are capable of sensing Zn^{2+} ions in biological samples. These new sensing platforms retain high selectivity of ion binding associated with a small molecule sensor, while also allowing sampling of small volumes and on/off switching. We have demonstrated that multiple measurements can be made on a single sample without the need to change the sensor. This is particularly attractive for biological experiments, where sample availability and volumes often limit the number of experiments that can be performed. More significantly, this work paves the way to a generic approach for developing surface-based ion sensors using a range of sensor molecules, which can be attached to a surface without the need for its chemical modification. This presents an opportunity for the development of new and highly specific ion sensors for real time sensing applications.

Supporting Information

The Supporting Information is available free of charge on the ACS Publication Website.

- SP2-liposome generation
- Preparation of SP2-maltose-liposomes
- Attachment of biosensor material to surface of suspended-core fiber
- Biological assays

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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