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Flexible Ag-C_{60} Nano-Biosensors based on Surface Plasmon Coupled Emission for Clinical and Forensic Applications

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Abstract

The relative low-sensitivity of fluorescence detection schemes, which are mainly limited by the isotropic nature of fluorophore emission, can be overcome by utilizing surface plasmon coupled emission (SPCE). In this study, we demonstrate directional emission from fluorophores on flexible Ag-C_{60} SPCE sensor platforms for point-of-care sensing, in healthcare and forensic sensing scenarios, with at least 10 times higher sensitivity than traditional fluorescence sensing schemes. Adopting the highly sensitive Ag-C_{60} SPCE platform based on glass and novel low-cost flexible substrates, we report the unambiguous detection of acid-fast *Mycobacterium tuberculosis* (Mtb) bacteria at densities as low as 20 Mtb/mm^2; from non-acid-fast bacteria (e.g., *E. coli* and *S. aureus*), and the specific on-site detection of acid-fast sperm cells in human semen samples. In combination with the directional emission and high-sensitivity of SPCE platforms, we also demonstrate the utility of smartphones that can replace expensive and cumbersome detectors, to enable rapid hand-held detection of analytes in resource-limited settings; a much needed critical advance to biosensors, for developing countries.

Keywords: surface plasmon coupled emission (SPCE); acid-fast; Mycobacterium tuberculosis; semen; biosensing; smartphone sensor

Introduction

The ability to assess the health status, disease onset, progression, and treatment outcomes for a patient, through inexpensive and non-invasive methods is one of the grand challenges in healthcare. Addressing such a grand challenge involves two aspects: i) identification of specific biomarkers that clearly identify a healthy or diseased state, and ii) development of economical non-invasive approaches to unambiguously detect and monitor these biomarkers without the need for sophisticated and expensive laboratory-based equipment. The early and rapid identification of disease markers would empower doctors to administer suitable treatment modalities to effectively treat or control the progression of a disease.

In this regard, fluorescence sensors (including enzyme linked immunosorbent assay or ELISA) are being widely implemented for the diagnosis of biomarkers associated with diseases (such as HIV, tuberculosis (TB)[1] and hepatitis), detection of DNA, blood, and other body fluids important for forensic applications.[2-4] Although fluorescence sensors are highly selective and easy to handle, their sensitivity is fundamentally limited due to their low signal collection efficiency (<1%) - a consequence of the isotropic nature of fluorescence. We and others have previously shown that the isotropic emission of fluorophores like Rhodamine B (RhB), when coupled with the surface plasmon resonance (SPR) modes of a thin metal film like Au and especially Ag,[5-7] through a protective spacer layer (traditionally ~5 nm thick SiO_2), results in the generation of highly directional and wavelength resolved emission.[8, 9] This phenomenon, known as surface plasmon coupled emission (SPCE) enables >50% signal collection efficiency, and thereby results in highly sensitive detection of biochemical-analytes.[5, 9, 10] Notwithstanding the excellent potential of SPCE technique for biosensing,[11, 12] its implementation as a handheld point-of-care (POC) device in resource-limited-settings (RLS) is still constrained by the following challenges: i) inefficient energy coupling between fluorophore and SPR modes through traditional SiO_2 layers, ii) the lack of flexible platforms and scalable ways to produce them, and iii) developing portable and inexpensive detection schemes.

Previously, we resolved challenge (i) by replacing traditional SiO_2 spacer layers with *active* nanocarbons (e.g., graphene and C_{60}) and demonstrated a 40-fold enhancement in the SPCE signal via "active" π–π interactions with the fluorophores (as opposed to *passive* SiO_2 layers which do not interact with the fluorophores).[6, 7, 10] In this study, we build on the success of these

nanocarbon spacers to: (a) fabricate inexpensive, scalable and flexible Ag-C_{60} SPCE (FLAG-C_{60}) substrates as sensing platforms, and (b) demonstrate their utility in smartphone based bio-sensors for clinical and forensic applications; especially for the low-cost, highly specific and rapid detection of: i) acid-fast *Mycobacterium tuberculosis* (Mtb) bacteria, which is the underlying cause for the high incidence of the fatal tuberculosis infection; over non-acid-fast bacteria like *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*), and ii) on-site detection of acid-fast sperm cells/semen samples; over saliva and urine. While our detection scheme is based on the presence of mycolic acid (stained with RhB) which is specific to the acid-fast Mtb and sperm cells; the low-cost FLAG-C_{60} platform amplifies the fluorescence signal; thereby facilitating the portable, rapid, and simplistic POC detection.

Materials and Methods

Fabrication of Ag-C_{60} Thin Films on Glass & Flexible SPCE Substrates

Ag thin films (~50 nm) were deposited on pyrex microscope slides (75×25 mm) and cellulose acetate flexible substrates (75×25 mm overhead projector films), using a home-built physical vapor deposition system.[6] About 50 mg of C_{60} powder (purchased from Sigma-Aldrich; 99.5% purity, CAS number 99685-96-8) was loaded into a W evaporation boat and sublimed onto the 50 nm thick Ag films. Based on our previous studies, C_{60} films of desired thicknesses (~10 nm) were prepared using an in-built shutter that was placed over the W boat.[7] Both Ag and C_{60} were deposited at 10^{-5} Torr and the thickness was regulated by a quartz-crystal film thickness monitor, to fabricate the final glass/flexible Ag-C_{60} (GLAG/FLAG-C_{60}) SPCE substrates (Fig. 1).

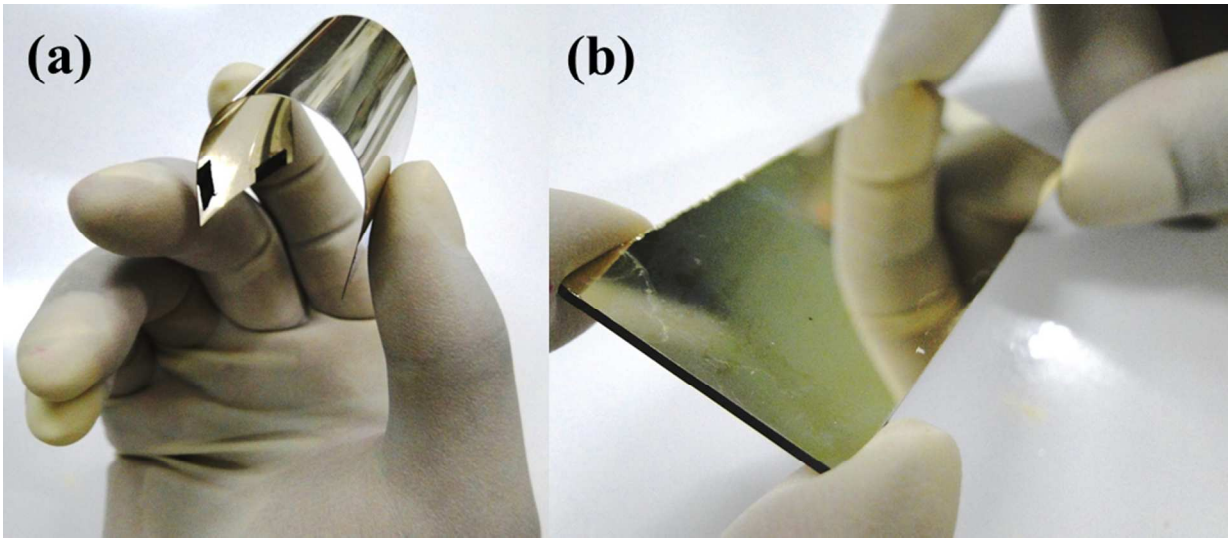

Figure 1 Photographs of (a) Ag-C_{60} flexible SPCE substrate (FLAG-C_{60}) and (b) Ag-C_{60} glass SPCE substrate (GLAG-C_{60}).

Fixing Acid-Fast and Non-Acid-Fast Bacteria on Ag-C_{60} SPCE Substrates

All the bacterial isolates used in this study were ATCC cultures and were procured from Sunshine Hospitals, Secunderabad, India. The following pure cultures were used: *Mycobacterium tuberculosis* (Mtb) ATCC 27294, *Escherichia coli* (*E. coli*) ATCC 25922 and *Staphylococcus aureus* (*S. aureus*) ATCC 25923. The non-acid-fast *E. coli* and *S. aureus* were cultured on a nutrient agar medium, while the acid fast Mtb was cultured on Löwenstein-Jensen medium (LJ medium). The handling of bacteria was performed inside a bio-safety cabinet under sterile conditions. Prior to fixing, all bacterial colonies were suspended in a saline solution, and were subsequently pipetted onto the GLAG-C_{60} and FLAG- C_{60} substrates. The bacteria were then heat fixed by gently running the substrates over a low flame.

Fixing Sperm Cells on Ag-C_{60} SPCE Substrates

Semen samples from healthy subjects were procured from Sunshine Hospitals, Secunderabad, India. The stock semen sample (~2×10^7 spermatozoa/ml) was diluted in distilled water (10% dilution) and subsequently drop-casted onto the GLAG-C_{60} and FLAG- C_{60} substrates. Sperm cells were gently heat fixed onto the substrates using a heating mantle. As semen is almost a colorless/translucent fluid, we also examined the fluorescence emission profile from other

colorless body fluids such as saliva and urine that were deemed as *controls*. These *controls* were fixed onto the Ag-C_{60} substrates in a manner similar to the semen samples.

Modified Acid-Fast Staining Protocol on SPCE Substrates

For SPCE measurements, we modified the traditional acid-fast staining protocol as elaborated below (Fig. 2). RhB, a previously reported good acid-fast dye was used as the fluorophore in our studies.[13] Solution of RhB was prepared in a mixture of water, phenol and glycerol (5:2:15). A 0.5% hydrochloric acid-ethanol (HCl-EtOH) mixture was prepared for the acid-treatment/decolorizing process. The substrates coated with bacteria, sperm cells and *controls* were initially heated to melt the mycolic acid layer of the acid-fast biological samples. The substrates were then flooded with the RhB solution and allowed to stand for three minutes. Excess RhB was washed off using distilled water and the biological samples were subsequently subjected to acid-alcohol treatment by flooding the slide with HCl-EtOH mixture for one minute. Following acid treatment, the slides were again washed with distilled water and air-dried. Importantly, further conventional steps that entail elaborate counter-staining with $KMnO_4$ and subsequent wash procedures in any acid-fast protocol were not needed as SPCE is a non-microscopic platform as elucidated below.

SPCE Measurements

Following the modified acid-fast staining protocol, the substrates containing bacteria, semen and *control* samples were affixed onto a hemi-cylindrical prism (Fig. 2). As in a typical SPCE setup,[5] the prism was mounted on a calibrated rotary stage (not shown in the figure), and a 532 nm c.w. laser (30mW) was used as the excitation source in the Reverse Kretschmann (RK) optical configuration, which we have depicted previously.[7] The SPCE signal (@580 nm) arising from the samples was recorded using a USB4000 Ocean Optics© fiber optic spectrometer; Ocean Optics, Florida, USA.

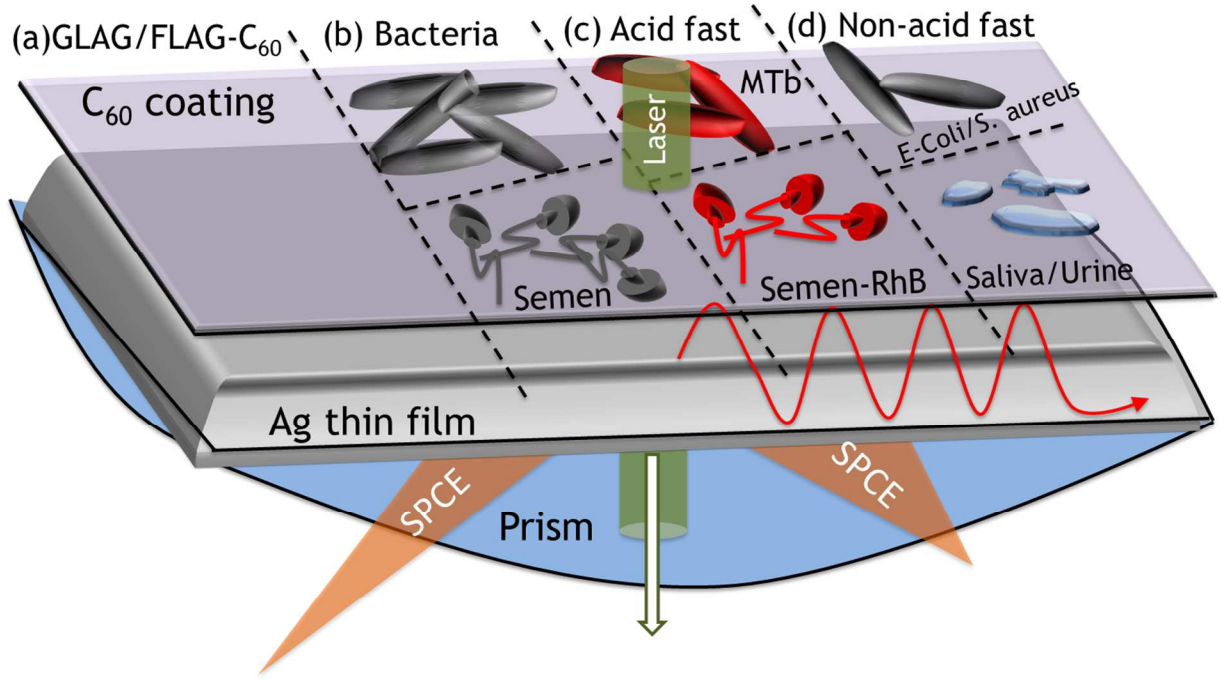

Figure 2 (a) A schematic depicting the use of GLAG/FLAG-C_{60} SPCE substrates in the Reverse Kretschmann (RK) optical configuration, for the detection of acid-fast bacteria (Mtb) and sperm cells. A laser beam directly excites the samples on the GLAG/FLAG-C_{60} substrates and the SPCE signal is observed through the hemi-cylindrical prism. Bacteria/semen samples are first coated (b), heat-fixed, and subsequently exposed to RhB. Acid-fact bacteria (Mtb) and sperm cells are resistant to acid-alcohol decolorizing and thus fluoresce strongly with a strong plasmon coupled signal (c); while non-acid fast specimens (d) *E. coli*, *S. aureus*, saliva, and urine do not exhibit any SPCE signal.

Fluorescence Microscopy

As stated above, we present SPCE as a non-microscopic tool for the specific detection of acid-fast bacteria (Mtb) and sperm (for clinical and forensic applications) using the samples prepared on GLAG/FLAG-C_{60} substrates (cf. Fig. 2). However, we have also performed fluorescence microscopy (Model BM-3000 FLT, LABEN Instruments, equipped with a Sony Cybershot© camera, DSC-HX1 series) on these samples to validate that the SPCE signals indeed originate from the acid-fast structures. Fluorescence microscopy also enables us to assess the sensitivity of the SPCE platform that can be deduced by observing the number of cells being detected in the view scope area.

Results and Discussion

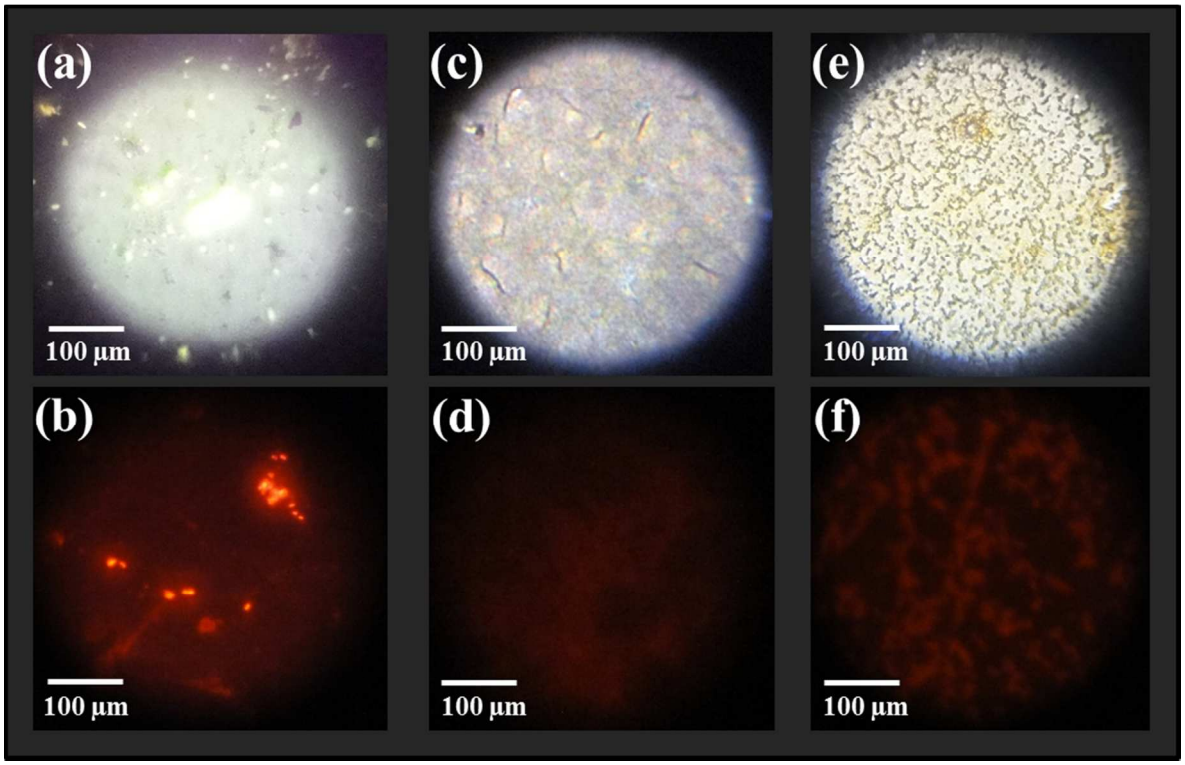

Figure 3 Photographs of the bacteria-coated AG-C_{60} substrates from fluorescence microscopy (top row) showing (a) Mtb cells, (c) rod shaped *E. coli* and (e) cocci clusters of *S. aureus* under white light illumination. The corresponding fluorescence photographs obtained using green laser excitation (λ=532 nm) are shown in the bottom row where; only the acid-fast Mtb cells display bright orange fluorescence (@580 nm) revealing its characteristic rod shape morphology (b), while no fluorescence is observed from the non-acid-fast *E. coli* and *S. aureus* bacteria; (d) and (f).

Traditionally, microscopic examination of acid-fast stained samples (Fig. 3) has been one of most widely implemented methods (as suggested by the Center for Disease Control, USA) for the diagnosis of TB. As evident in Fig. 3b, only acid-fast Mtb exhibits the reddish-orange fluorescence upon staining, unlike the *E. coli* and *S. aureus*, which confirmed the high specificity of the acid-fast staining procedure. This specificity is due to the presence of the long chain mycolic acid in the cell wall of Mtb. On performing heat fixation (see experimental section), the layer of mycolic acid in Mtb denatures thereby allowing RhB molecules to penetrate its cell wall.

Subsequent cooling enables the mycolic acid layer to regain its integrity and resist decolorizing caused by acid-alcohol treatment. The already penetrated RhB molecules are retained within the cell wall of the acid-fast Mtb, enabling it to fluoresce. On the contrary, the non-acid-fast *E. coli* and *S. aureus* bacteria, though present on the microscope slides (Figs. 3c and 3e), do not contain mycolic acid in their cell walls and are hence easily decolorized upon acid-alcohol treatment, and do not fluoresce upon excitation with the green laser (Figs. 3d and 3f). Another important factor discernible from Fig. 3 is the difference in the density of, *E. coli* and *S. aureus* bacteria, which is vastly greater than that of Mtb. This is attributed to the well-known cell wall composition of the acid-fast microorganisms that contains high concentration of lipids and fatty acids (mycolic acids), which possess a waxy character and hence exhibit a low coefficient of friction. Thus, acid-fast organisms such as Mtb usually wash off a substrate more easily than the non-acid fast bacteria.

While the microscopic examination (Fig. 3) is highly specific and reveals the presence of Mtb, an early diagnosis of tuberculosis in RLS setting is still challenging due to the significantly lower number of Mtb observed in sputum samples that results in either a weak or no signal in fluorescence microscopy. In this context, a chest X-ray scan and culturing of bacteria from sputum samples are prescribed, to confirm whether or not the patient is infected by the Mtb bacteria. However, the culturing usually takes a few weeks due to the extremely slow doubling rate of Mtb (16-20 hours per division), thereby subjecting the patient to an increased risk of disease progression. Moreover, fluorescence microscopes and X-ray scanners are often unavailable in RLS settings of low-and-middle income countries. In such circumstances, FLAG-C_{60} SPCE substrates can facilitate the rapid and sensitive detection of Mtb even at a low concentration (as low as 20 Mtb/mm^2) without the need for expensive infrastructure.

In SPCE, the observed fluorescence intensity ($I(\lambda)$) from molecules/fluorophores present near the nanostructured silver surface can be described as $I(\lambda) = L^2(\lambda)*Z(\lambda)$, where $L^2(\lambda)$ accounts for the local enhanced electromagnetic field intensity due to the surface plasmons in the metal, and $Z(\lambda)$ describes the relative radiative yield of the excited fluorophore. The coupling between the molecular dipoles in the fluorophore and the metal plasmons can enhance the radiative yield only when the fluorophore is at an optimal distance d_{en} from the metal surface. Accordingly, a passive spacer layer, which does not influence $Z(\lambda)$, such as SiO_2 is traditionally used in SPCE substrates.[5] We have previously shown that nanocarbon spacer layers, such as graphene and C_{60},

can improve the relative radiative yield through π–π and multipolar interactions with the fluorophores and thereby act as an *active* spacer layer (i.e., provide an additional enhancement over traditional SPCE by improving $Z(\lambda)$).[6, 7] While all nanocarbon allotropes (viz., graphene, carbon nanotubes, fullerenes, and carbon nanodots) could act as active spacer materials, some nanocarbons such as carbon nanodots possess strong intrinsic fluorescence that result in false-signals while other nanocarbons (graphene and nanotubes) are not amenable for producing uniform coatings directly on Ag films. Thusly, as we had demonstrated earlier, C_{60} is the most practical spacer material as it can: i) be directly sublimed from its molecular solid form on to Ag films with excellent thickness control, ii) facilitate pi-orbital based, organized orientation of fluorophore molecules for effective emission dipole-plasmon alignment and iii) enable highly efficient photo-induced charge transfer from excited state of RhB to the plasmon modes of Ag.[7] In light of these advantageous attributes, in our design, the fluorescence from the stained analytes (i.e., Mtb and sperm) is coupled, via C_{60}, to the Ag SPR modes, for sensing with high signal to noise ratios.

As shown below in Fig. 4, we recorded the RhB emission (at 580 nm), arising from the bacteria coated GLAG/FLAG-C_{60} substrates upon 532 nm excitation. The high-specificity of acid-fast staining is clearly evident in the absence of any fluorescence signal from *E. coli* and *S. aureus* samples (Fig. 4a) concurring with the microscope images discussed in Fig. 3. As expected, the recorded SPCE signal is highly directional (Fig. 4b) due to the strong coupling between RhB emission from stained bacteria and the SPR modes in the Ag thin film. More importantly, the SPCE signal is ~10 fold greater than the free space signal, (not coupled to SPR modes), suggesting at least a ten-fold increase in the sensitivity compared to the observation of isotropic fluorescence. Furthermore, we obtained a distinct SPCE signal for Mtb samples even when the stock Mtb solution was reduced to 25% dilution for coating the GLAG/FLAG-C60 slides (Fig. 4a). Returning to our microscopy data (cf. Fig. 3), we evaluated the number density of Mtb cells immobilized on GLAG/FLAG-C60 slides, (using stock solution), after acid-fast staining, to be around 90-100 Mtb/mm^2. Considering the area of a typical laser spot (used as the excitation source in SPCE) to be around ~0.78 mm^2 (an equivalent diameter of 1 mm), and approximating the 25% dilution to yield roughly 20-25 Mtb/mm^2; the detection limit of the GLAG/FLAG-C_{60} platform is at least ~20 Mtb/mm^2. This level of sensitivity is highly significant as clinical sputum samples of patients infected with TB in early stages usually contains about 10-100,000 Mtb

cells/ml[14] resulting in a Mtb density of >>20 Mtb/mm^2, that can be promptly and unambiguously detected using these GLAG/FLAG-C_{60} platforms.

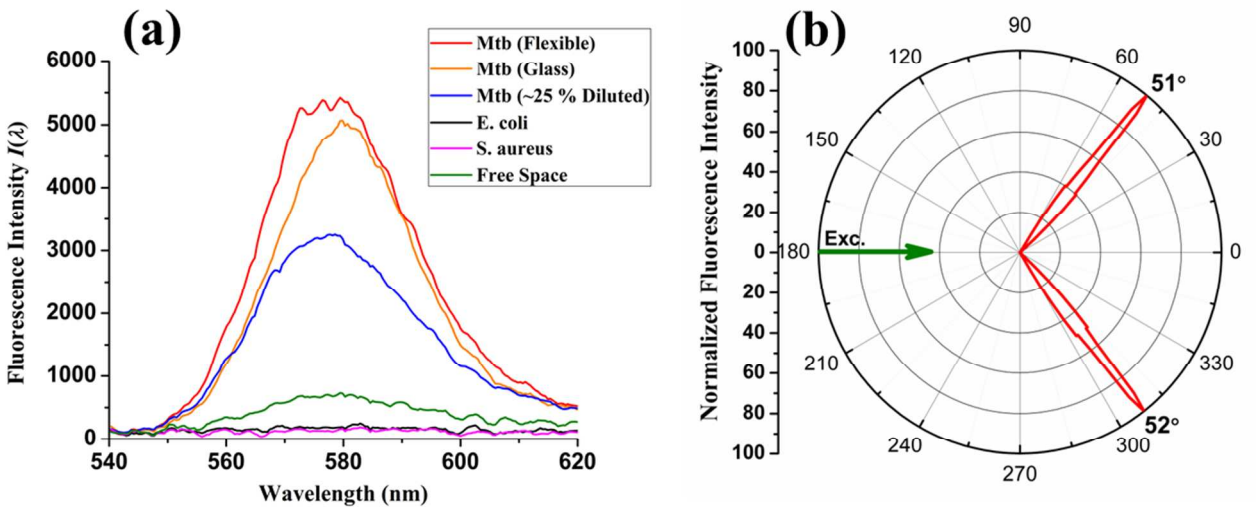

Figure 4 (a) A plot depicting the fluorescence intensity of acid-fast Mtb bacteria on the GLAG/FLAG-C_{60} substrates versus wavelength. Also, depicted in the plot are fluorescence signals from non-acid-fast *E. coli* and *S. aureus* coated substrates which exhibit negligible intensities. (b) Highly directional angular distribution of the emission signal around the normal of the prism, which is a characteristic feature of SPCE.

As discussed in the introduction, we also fabricated FLAG-C_{60} substrates to enable flexible sensing platforms for device fabrication, which would be useful in POC/RLS settings. Interestingly, we found the SPCE signal intensity of the FLAG-C_{60} substrates to be superior over the GLAG-C_{60} substrates (Fig. 4a). We attribute this finding to the robustness of the FLAG-C_{60} substrates, in which the Ag-C_{60} coat was impervious to multiple wash procedures, while Ag-C_{60} coat occasionally peeled off during the acid wash procedures. Importantly, the relative high surface roughness and higher surface energy of the cellulose acetate flexible substrates, compared to the glass substrates, enables more efficient binding of a greater number of RhB fluorophore molecules to the FLAG-C_{60} coat, resulting in greater signal-to-noise ratios, as reflected in our studies; which also renders these substrates to become impervious to chemical washes and device integration.

Detection of Sperm Cells on Ag-C_{60} SPCE Platform: The detection of semen samples is important for forensic investigations pertaining to sex-crimes. The standard blue or ultraviolet light (Wood's Lamp) test, which is often used to visualize semen on-site, is however not unique

to semen alone, and could show false positive signals by responding to many other natural and synthetic molecules in body fluids that fluoresce under ultra-violet illumination.[15, 16] In this context, similar to bacterial detection described in Fig. 3, we performed fluorescence microscopy on the acid-fast stained semen coated Ag-C_{60} substrates, which is a traditional confirmatory test that uses the presence of mycolic acid in the head of sperm cells, to unambiguously identify semen from other biological fluids of the body, (e.g. saliva and urine).

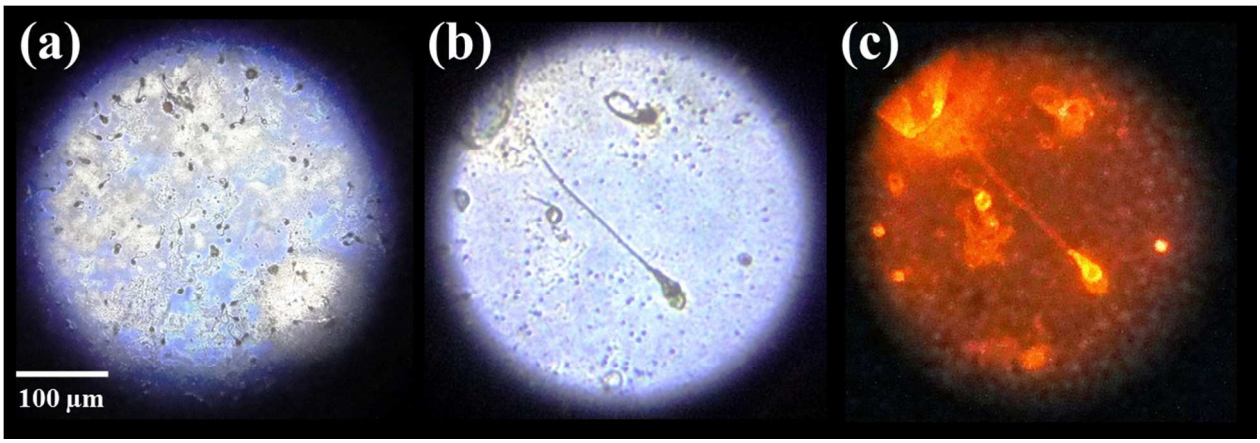

Figure 5 Photographs of sperm cells on Ag-C_{60} substrates under fluorescence microscope showing: (a) Numerous sperm cells under white light; (b) Zoomed in area of the substrate under white light (no scale-bar) showing sperm cells with distinct head and tail morphology; and (c) Photograph of zoomed area of sperm cells on green laser excitation (no scale-bar), exhibiting orange fluorescence distinctly arising from the acid-fast heads that corresponds to the SPCE emission @580 nm.

Fig. 5a shows the representative microscope image of a semen sample containing thousands of sperm cells on a glass substrate. The head-tail morphology of sperm cells in these semen samples is evident from both the white light and fluorescence images shown in Fig. 5b and c respectively. Notably, only the head of the sperm cell (Fig. 5c) exhibits orange fluorescence emission from RhB molecules, due to the presence of mycolic acid only in the head of sperm cells. Although microscopy is a good confirmatory test for the identification of semen samples, it is often performed in the lab and is time-consuming involving the use of cumbersome infrastructure, generally not available in RLS. In addressing this issue, here we demonstrate the advantage of using the economical and simplistic GLAG/FLAG-C_{60} SPCE platform for the on-site rapid

detection of semen, clearly differentiated from other body fluids like saliva and urine deemed as *controls* in this study.

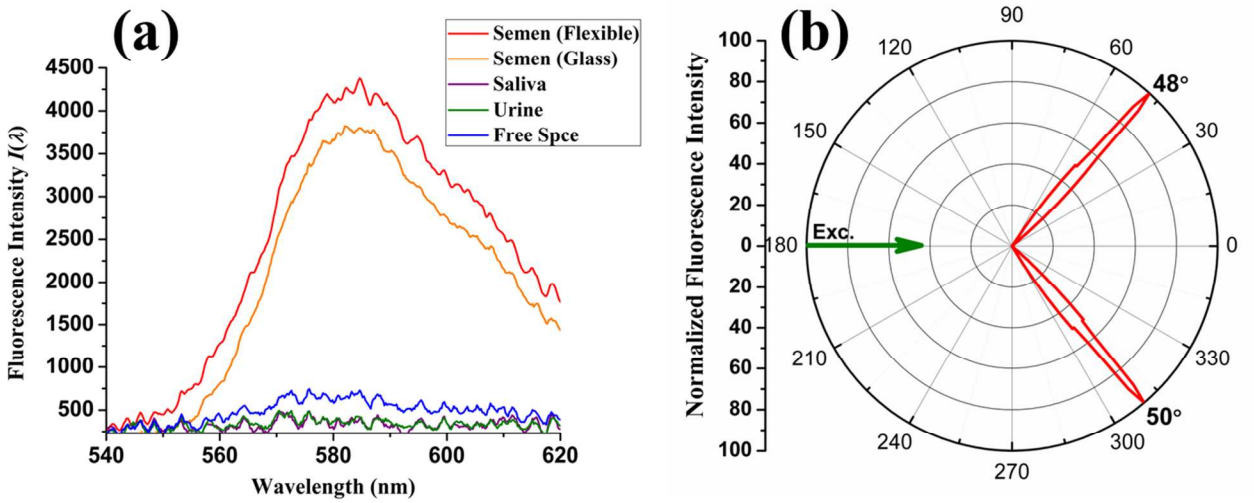

Figure 6 (a) A fluorescence intensity plot depicting the specific detection of acid-fast sperm cells in a semen sample, versus *control* samples (saliva and urine) on GLAG/FLAG-C_{60} substrates. (b) Highly directional angular emission profile of SPCE around the normal of prism.

As shown in Fig. 6a, semen/sperm samples exhibited strong fluorescence after acid-fast staining, while saliva and urine, (which do not contain mycolic acid), did not exhibit any signal rendering SPCE an ideal portable forensic sensing platform. As observed in the case of Mtb, the SPCE signal intensity was superior (~10 fold) to that of free space emission, and also highly directional as depicted in Fig. 6b. Furthermore, the FLAG-C_{60} substrates exhibited a greater sensitivity for the detection of semen/sperm, akin to Mtb detection. On microscopic evaluation (as described earlier) we were able to detect ~10 sperm-cells/mm^2, which is extremely sensitive. This is beneficial, as the FLAG-C_{60} substrates can be directly used as swabs to collect biological fluid samples, enabling the immediate and prompt detection of sperm cells even from low sample volumes. Such a rapid detection of semen directly on-site is significant because, most of the currently used tests for on-site semen detection are highly presumptive.[15-17]

Smartphone based Sensing of SPCE Signals: Having established the applicability of the novel flexible Ag-C_{60} SPCE bio-sensing platform for clinical and forensic sensing scenarios; we have also successfully demonstrated the proof-of-concept extension of a smartphone, for the detection of SPCE signals from micromolar concentration of fluorophores used in our studies; (See Supporting Information). This opens up the prospect of hand-held sensing of SPCE signals

where expensive spectrometers and detectors can be replaced by simplistic mobile phones as POC devices.

Conclusions: We successfully demonstrated the use of flexible and inexpensive SPCE platforms for the rapid and sensitive detection of Mtb and semen, for clinical and forensic POC sensing in RLS settings; without the need for sophisticated microscopic evaluation or cumbersome optics. The ability to detect Mtb at an early stage (~20 Mtb/mm^2), and rapid on-site specific detection of semen using the GLAG/FLAG-C_{60} substrates are critical advances, because current fluorescence-based methods are limited due to the inherent low-sensitivity associated with isotropic fluorescence.

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