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Smartphone based bacterial detection using biofunctionalized fluorescent nanoparticles

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Abstract We are describing immunochromatographic test strips with smart phone-based fluorescence readout. They are intended for use in the detection of the foodborne bacterial pathogens *Salmonella* spp. and *Escherichia coli* O157. Silica nanoparticles (SiNPs) were doped with FITC and Ru(bpy), conjugated to the respective antibodies, and then used in a conventional lateral flow immunoassay (LFIA). Fluorescence was recorded by inserting the nitrocellulose strip into a smart phone-based fluorimeter consisting of a light weight (40 g) optical module containing an LED light source, a fluorescence filter set and a lens attached to the integrated camera of the cell phone in order to acquire high-resolution fluorescence images. The images were analysed by exploiting the quick image processing application of the cell phone and enable the detection of pathogens within few minutes. This LFIA is capable of detecting pathogens in concentrations as low as 10^5 cfu mL^{-1} directly from test samples without pre-enrichment. The detection is one order of magnitude better compared to gold nanoparticle-based LFIAs under similar condition. The

successful combination of fluorescent nanoparticle-based pathogen detection by LFIAs with a smart phone-based detection platform has resulted in a portable device with improved diagnosis features and having potential application in diagnostics and environmental monitoring.

Introduction

Constant improvements are being made in diagnostic tests to obtain sensitive, specific, user friendly, low cost and reliable methodology under field conditions in resource limited situations [1]. Simple immunoassay protocols have become increasingly common at resource – limited sites, particularly in the form of lateral flow immunoassay (LFIA) for testing pregnancy, drug of abuse, diagnosis of various diseases and environmental monitoring [2]. However, the inherent limitation of traditional LFIA towards pathogen detection in terms of low sensitivity has necessitated research in the development of alternative labels to improve the detection limit of the assay [3]. In the past, several methods with different fluorescence labels have been tested to increase the sensitivity [4–7]. However, the direct use of flurophores has certain limitations such as photo bleaching, pH-dependence of the fluorescent signal or limited fluorescence intensity. To overcome these limitations, this study employs successful use of dye doped silica nanoparticles (SiNPs) as signaling probes which gives strong signal and have improved stability. In addition to this, wide compatibility of silica with biological materials and ease in biofunctionalization [8] render them ideal candidate for such applications. To note, manual interpretation of LFIA signal could be challenging for the untrained users who attempt to interpret the results. Hence, LFIA demands the development

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of a simple user friendly device to fully utilize its benefits. Earlier attempts to carryout quantitative detection of conventional LFIA signal have led to the use of special kind of reflectance photometer, necessitating skilled manpower and complex instrumentation [9]. It is pertinent to note that, over the past few years there has been consistent effort by various research groups [10–12] and commercial companies (Urisys 2400 and Miditron, Roche Diagnostics) to develop specialized assays and dipstick devices which led to increased cost and time. To address this gap, the available smart phone applications are utilized for pathogen and various biomarker diagnoses [13–17]. Smartphone cameras have been previously shown to be a portable microscope for monitoring pathogens, inexpensive spectrophotometer for educational purpose [18, 19]. Ozcan research group has demonstrated a prototype diagnostic test device on android platform for rapid detection of gold nanoparticles signal in LFIA [20]. In this scenario, the work presented here compliment the utilization of smart phone for interpretation and quantification of fluorescence LFIA signal using simple optical module attached to the camera of the smart phone. A novel approach was introduced combining sensitivity of fluorescence LFIA assay with portability of smart phone based device for point – of – care detection of food borne pathogen.

Experimental

Bacterial strains and reagents

The *Salmonella* spp. *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Proteus mirabilis* and *Pseudomonas aeruginosa* reference strain was obtained from Microbial Type Culture Collection, India. The 10 strains of *Salmonella* spp. isolated from typhoid fever patients were obtained from Sundaram Medical Foundation hospital laboratory. The clinical isolate of pathogenic *Escherichia coli* O157 was obtained from Christian Medical College, Vellore, India. All the organisms were cultured on nutrient broth at 37 °C, 195 rpm for 16–18 h. All the reagents were of analytical grade and purchased from Sigma (www.sigmaaldrich.com).

Synthesis of dye doped silica nanoparticles

The FITC doped SiNPs were prepared by Stoeber's method [21]. Briefly, about 6 mg of FITC was dissolved in 2 mL of ethanol and 160 µL of 3-aminopropyltrimethoxysilane was added under magnetic stirring. To that was added 150 µL of tetraethyl orthosilicate, distilled water (1 M) and the mixture was made to 20 mL with ethanol followed by addition of 50 µL of ammonium hydroxide. Reaction was allowed to proceed overnight at room temperature under magnetic

stirring resulting in silica encapsulated FITC nanoparticles. The obtained nanoparticles were washed several times with ethanol, vacuum dried and stored at room temperature. The Ru(bpy) doped SiNPs were synthesized by microemulsion method [22]. Briefly, 7.5 mL of cyclohexane was added to 1.8 mL of n-hexanol and 1.77 mL of Triton X-100 followed by addition of 480 µL of 20 mM Rubpy dye in water and mixed vigorously to form a microemulsion. About 100 µL of tetraethyl orthosilicate was added to above mixture under magnetic stirring. The reaction mixture was stirred continuously for 20 min followed by addition of 60 µL of 30 % ammonium hydroxide, and stirring continued for next 24 h at room temperature. After this period, an equal amount of acetone by volume of the reaction mixture was added and vortexed vigorously to break the microemulsion. The solution was centrifuged at 3,000 xg for 10 min and the particles obtained were washed several times with vortex and sonication in 95 % ethanol. The final particles were dried in vacuum and stored at room temperature for later use. The particles were characterization by SEM, fluorescence spectroscopy and FT – IR spectroscopy (Supplementary data).

Biofunctionalization of nanoparticles

The anti – *Salmonella* monoclonal antibody was developed against whole cell *Salmonella* antigen and conjugated to dye doped SiNPs as described previously by our group [23]. About 30 mg of nanoparticles were reacted with 20 mL of 1 % aminopropyl trimethoxysilane in 1 mM acetic acid under continuous mechanical stirring for 30 min. The particles were allowed to react with 10 % succinic anhydride in dimethylformamide for overnight at room temperature under nitrogen atmosphere. After incubation, the obtained carboxyl modified dye doped SiNPs were washed three times with water. Then 1 mL of 1 mg.mL^{-1} 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide and N- Hydroxysuccinimide in water was added and incubated at room temperature for 30 min. The activated nanoparticles were then immobilized with 30 µg of anti - *Salmonella* monoclonal antibody for 24 h at 4 °C in a rotary shaker. After washing thrice with PBS buffer, the free N-Hydroxysuccinimide esters were blocked by adding 40 mM ethanolamine and 1 % Bovine serum albumin in 1 mL of PBS for 2 h at room temperature. The resulting antibody conjugated dye doped SiNPs were resuspended in 1 mL of phosphate buffered saline containing 0.1 % Bovine serum albumin at 4 °C until further use. Similar protocol was followed for conjugation of *Escherichia coli* O157 polyclonal antibody (Kirkegaard & Perry Laboratories Inc, www.kpl.com) to the dye doped SiNPs. The preparation of antibody conjugated gold nanoparticles was carried out on 20 nm colloidal gold nanoparticles (Sigma, www.sigmaaldrich.com) using the previously described procedure [24].

Assembly of test strip

The test strip (75×3 mm dimension) containing nitrocellulose membrane with pore size of 10 μm, absorbent pad, conjugate pad and sample pad required for dipstick preparation were purchased from Advanced Microdevices Pvt Ltd (www.mdimembrane.com). Initially, the capture antibody was immobilized in the designated test region and the control region of the nitrocellulose membrane [24]. LFIA relies on the migration of liquid across the surface of the nitrocellulose membrane. The bacteria present in the test sample reacts with the antibody conjugated nanoparticles in the conjugate pad which is placed beneath the sample pad and the immune complex were trapped in the test line immobilized with capture antibody to result in the fluorescent signal. The free antibody conjugated nanoparticles further move and gets trapped in the control line containing the anti-goat IgG for *Escherichia coli* O157 test strip and anti- mouse IgG for *Salmonella* test strip. The control line was used to confirm active migration across the membrane. The fluorescence signal caused by the accumulation of dye doped silica nanoparticles in the test line and control line provided the basis for detection of the pathogen. For immobilization in the test strip, 100 μL (1 mg.mL^{-1}) of antibody was mixed with 8 μL of 20 % sucrose solution prepared with 50 mM potassium dihydrogen phosphate buffer (pH 7.5) and 8 μL of isopropanol. About 1 μL spot of *Salmonella* polysera and goat anti – mouse IgG (Bangalore Genei, www.bangaloregenei.com) was used for immobilization in the test line and the control line respectively for preparation of *Salmonella* LFIA strip. After immobilization of antibody, the membrane is blocked with 1 % Bovine serum albumin in 50 mM boric acid buffer (pH 8.5) for 30 min at room temperature to prevent non-specific binding. After incubation, the membrane was washed twice with PBS containing 0.05 % Tween 20. The test strips were allowed to dry at room temperature for 12 h. About 2 μL of antibody conjugated dye doped SiNPs was added to the conjugate pad and allowed to dry for 5 min. The adsorbent pad and the conjugate pad were mounted on an adhesive coated backing sheet. The prepared LFIA strip was stored in air tight container at 4 °C until use. Similarly, *Escherichia coli* O157 polyclonal antibody and rabbit anti-goat IgG was immobilized in the test line and control line respectively for preparation of *Escherichia coli* O157 LFIA strip.

Construction of fluorescence device

To enable the utility of fluorescence LFIA in low-resource settings, a simple optical design (Fig. 1a) was assembled. It consists of 3.7 V battery powered LED light source (Osram, in.rsdelivers.com) for illumination, a fluorescence filter cube (Chroma, www.chroma.com), a plano convex lens (Thorlabs, www.thorlabs.us) of 50 mm focal length and a sample tray for inserting the test strip. The band pass filter (460–490 nm) allows excitation of both dyes and also eliminate the high wavelength tail of the LED emission. The barrier filter (520 nm) enable simultaneous observation of fluorescence signals from both dye doped SiNPs without the need to change the filter set combinations, allowing multiplex detection. However, the module is designed to enable easy exchange of the fluorescence filters if required. The optical system is enclosed in a light proof plastic box to ensure improved signal to noise ratio by reducing ambient light. The entire optical module weighing ~40 g (Fig. 1b) is attached in front of a camera (5 mega pixel) of a smart phone (Sony Live with Walkman) to capture the fluorescence signal from LFIA strip. As observed in Fig. 1c, selected region of interests were analyzed using smartphone resident application which exhibited fluorescence intensity maxima corresponding to the test and control spots of the LFIA strip.

Fluorescence lateral flow immunoassay

The overnight grown cells were serially diluted in sterile PBS to attain 10^7– 10^4 cfu.mL^{-1}. The samples were centrifuged at 8,000 xg for 2 min and resuspended in 200 μL of sterile PBS. The 200 μL was determined to be the optimum running volume required for the assay. For evaluation, all the samples were taken in 96 well microtiter plates. The test strip was placed sample pad end down into the well allowing at least 10 min for adequate absorption and migration of the liquid along the strip. The strips were then inserted into the strip tray of the smartphone attached fluorescence LFIA device for image capture. The reproducibility of the assay was analysed by performing the test in triplicates. Subsequently, the assay was performed on 10 clinical isolates of *Salmonella* spp. to demonstrate the applicability in clinical diagnosis. The fluorescence LFIA results were further compared with conventional gold nanoparticles based LFIA and the sensitivity of the assay was evaluated. The specificity of the assay was determined against *Escherichia coli* O157*, Pseudomonas aeruginosa, Proteus mirabilis*, *Citrobacter freundii* and *Klebsiella pneumoniae*. Adopting the same procedure the detection limit and cross reactivity of the *Escherichia coli* O157 LFIA strip was determined against *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella* spp*, Citrobacter freundii, Proteus mirabilis* and *Pseudomonas aeruginosa*.

Image processing

The total intensity of the images obtained from the fluorescence LFIA strip was analyzed using an application in smart phone. The images were processed for the region of interest covering the test and control spot using Photo Editor Ultimate, downloaded from Google Play [25]. The processed images

Fig. 1 Smart phone based fluorescence LFIA device. **a)** Optical layout **b)** Snap-shot of the device **c)** Representative image showing the readout of the LFIA strip from the smartphone application. [1. Smartphone; 2. Planoconvex lens; 3. Fluorescence filter set; 4. Light source; 5. Light-proof box; 6. LFIA strip; 7. Strip tray
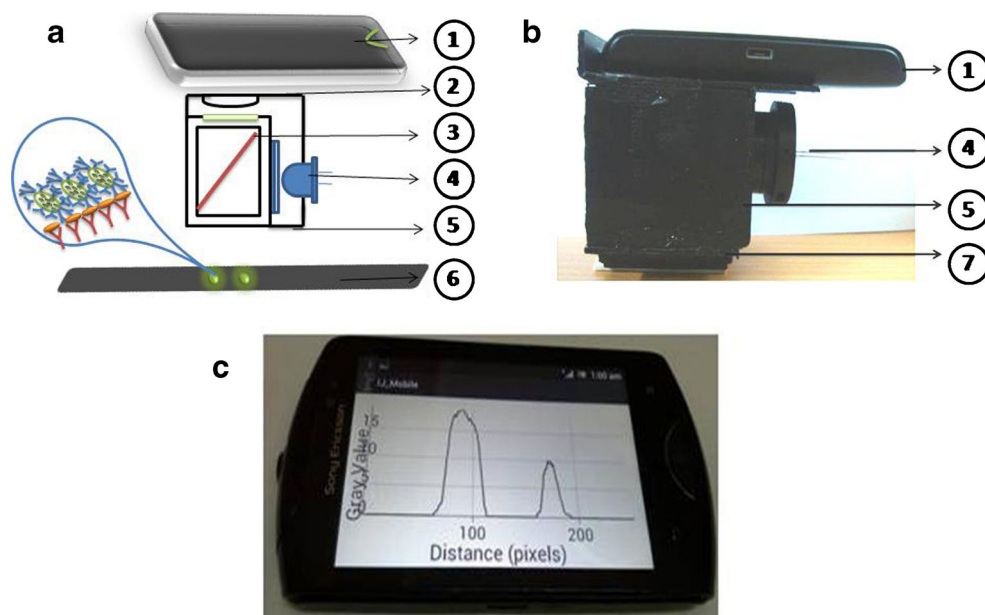
were saved and recalled in IJ_ mobile application [26] installed in the smart phone for analysis. The application provides the graphical readout of the total intensity profile along the region of interest. For comparison, the captured fluorescence images were also copied to a laptop and independently analyzed using ImageJ (Supplementary data). A rectangular region of interest was placed covering both the test and control spot to obtain the integrated fluorescence intensity using ImageJ.

Result and discussion

The organic dye FITC and the inorganic dye Rubpy doped silica nanoparticles were synthesized by Stoeber's and microemulsion methods respectively. The synthesized dye doped SiNPs were analysed by SEM to study the morphology and size of the nanoparticles. As observed in Fig. 2a and b, well dispersed and spherical nanoparticles in the size range of ~150 nm and ~100 nm for FITC and Ru(bpy) doped SiNPs respectively were obtained. Spectrofluorometric measurements of the dye doped SiNPs show emission at 525 nm for FITC doped SiNPs and 625 nm for Ru(bpy) doped SiNPs upon excitation at 475 nm in aqueous solution (Supplementary data).

Salmonella detection

The affinity of the antibody is essential for efficient binding of the cell–SiNPs immunoconjugate in the LFIA strip. The nanoparticles conjugated with mouse monoclonal antibody to *Salmonella* spp. were incubated with the *Salmonella* cells. The SEM image shows that nanoparticle conjugates adhered

to the surface of *Salmonella* spp. (Fig. 3a and b). Thus the developed antibody was found specific to the surface antigens of the whole cells. In addition, numerous dyes doped SiNPs were found bound to single bacterium which help improve the sensitivity of detection.

To determine the detection limit of the assay, the prepared fluorescent LFIA strips were tested on known concentration of *Salmonella* spp. and the signal was obtained through optical module attached to the smart phone. The red/green fluorescence signal could be detected by visual inspection of the image obtained from the smart phone device. Subsequently, the images of the fluorescence LFIA strips were analyzed using the smartphone resident application that readout the total intensity along the region of interest. As shown in Fig. 4, significant intensity peak was recorded from the test spot for sample containing $\geq 10^5$ cfu.mL^{-1} of bacteria. Consistent results were obtained during repeated screening ($n=3$). The fluorescence LFIA strip can detect the pathogens in the sample within few minutes using the simple attachment to the smart phone. Thus the smart phone based fluorescence diagnosis can be a simple, user friendly screening tool in the hands of a end user. Further, the captured fluorescence images were copied on to a laptop and the total intensity along the width of the spot was measured using ImageJ software for comparison. Observation of similar intensity profile (Supplementary data) validates the reliability of smart phone based measurements. The optimized assay was then validated on 10 independent clinical isolates of *Salmonella* spp. using the similar protocol. Successful detection was observed for all the 10 samples when assayed at the concentration of 10^5 cfu.mL^{-1}.

Further, the specificity of *Salmonella* fluorescence LFIA strips was determined against *Escherichia coli* O157, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Citrobacter freundii*

Fig. 2 SEM image of **a)** FITC doped silica nanoparticles by Stoeber's method **b)** Ru(bpy) doped silica nanoparticles by microemulsion method
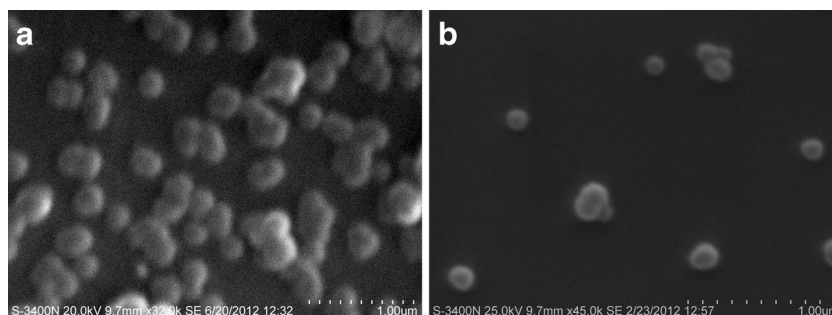
and *Klebsiella pneumoniae* at 10^5 cfu.mL^{-1}. There was no significant fluorescence observed in the test spot proving zero cross reactivity of the assay. As observed in Fig. 5, the *Escherichia coli* O157 LFIA also showed successful detection of *Escherichia coli* O157 from samples containing $\geq 10^5$ cfu.mL^{-1} and zero cross reaction was observed when tested against six negative strains (Supplementary data). The above results convincingly prove that the fluorescence LFIA strip can constitute a sensitive and specific diagnostic platform for bacterial pathogens.

To demonstrate the improved detection limit of fluorescence LFIA strips, conventional gold nanoparticles based LFIA assay was performed under similar setting. A visible red spot could be seen in the LFIA strips for sample containing $\geq 10^6$ cfu.mL^{-1} of *Salmonella* spp. and *Escherichia coli* O157 (Supplementary data). Despite the gold nanoparticles based LFIA strip is being simple and the results can be readily read with the naked eye, they are one order of magnitude less sensitive in detection. Usually, sensitivity in gold nanoparticle assay is increased by silver enhancement, aptamers or modified assay probes. However, this leads to increased cost and time [27, 28]. Multiplex PCR has been reported for rapid and sensitive detection of food borne pathogens [29]. However, a short pre-enrichment of the test samples are needed for reliable assay performance and to enable direct sample detection [30]. The developed fluorescence nanoparticles based LFIA could detect pathogen at levels of 10^5 cfu.mL^{-1} without pre-enrichment of the samples. It has been reported that ingestion of 10^5 organisms cause enteric fever in healthy individuals

[31]. The developed assay is well within this limit and hence can be a potential and rapid screening tool for detection of bacterial pathogens directly from the test sample to ensure food safety. The study demonstrates that both organic and inorganic dyes can be utilized as probes for detection of pathogens. The dye leakage from the silica nanoparticles is prevented by covalent attachment of the organic dye like FITC to the silica matrix by using the FITC-APTES as precursor during synthesis of dye doped SiNPs by using Stoeber's method. To note, for the synthesis of inorganic dye like Ru(bpy) doped SiNPs, which possesses a mainly hydrophilic nature, the reverse microemulsion method is used to enable a more efficient dye encapsulation without dye leakage during subsequent handling of the nanoparticles. This increased sensitivity of bacterial detection by fluorescence LFIA strip is due to the fact that (i) very high ratio of dye are encapsulated inside the silica shell and (ii) numerous antibody conjugated nanoparticle probes bind to the surface of the single bacterium. The strong fluorescence signal from dye doped SiNPs can be easily excited with a simple diffused LED and captured using a smart phone or digital camera [32]. The affinity of the antibody determines the variability of the assay on different test samples. The monoclonal and polyclonal antibody may behave differently. However, in accordance to our assay consistent results were observed on repeated ($n=3$) screening. Thus, our smart phone based fluorescence LFIA device satisfies the user friendly and portable screening applications without compromising on the sensitive detection.

Fig. 3 SEM imaging showing the binding of *Salmonella* spp. to **a)** FITC doped silica nanoparticles **b)** Ru(bpy) doped silica nanoparticles
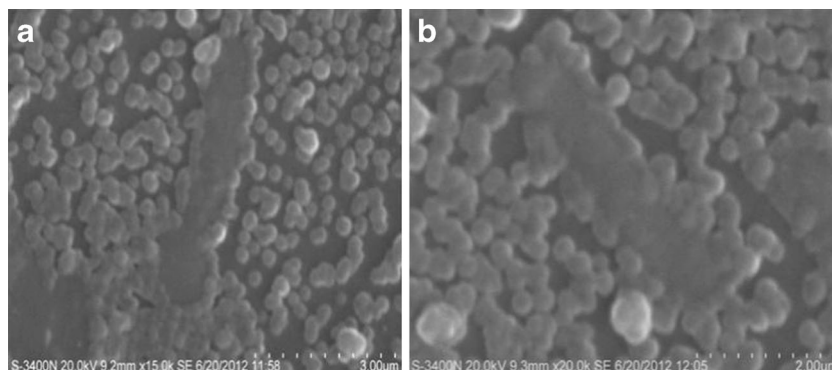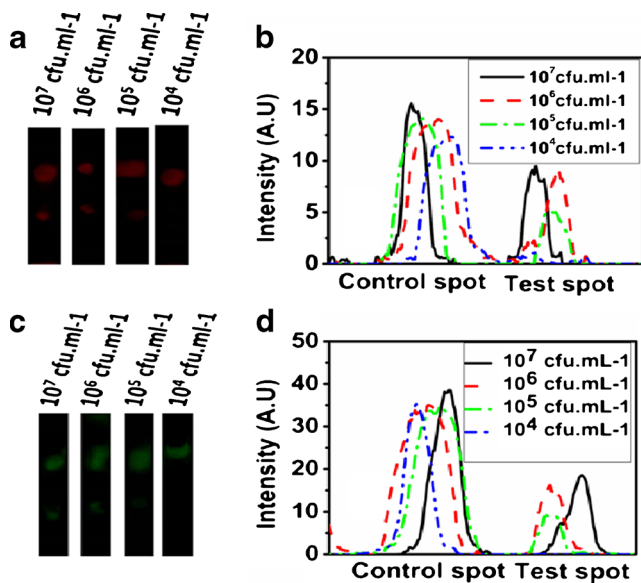
Fig. 4 Detection of *Salmonella* spp. using fluorescence LFIA strip. **a** and **c)** Snapshot of image captured using smartphone based device. **b** and **d)** Intensity plot obtained using smartphone resident application

Researchers have developed a rapid diagnostic test device to process the raw image of the gold nanoparticles based LFIA strip using a dedicated application running on the smart phone [16]. Recent reports also demonstrate a low cost fluorescent lateral flow assay [33, 34]. There have been efforts to establish computer screen shot assisted sensing using web/digital cameras for fluorescence biosensing [35]. In this work, we successfully demonstrate for the first time, the use of smart phone for both fluorescence image capture and its analysis. This can
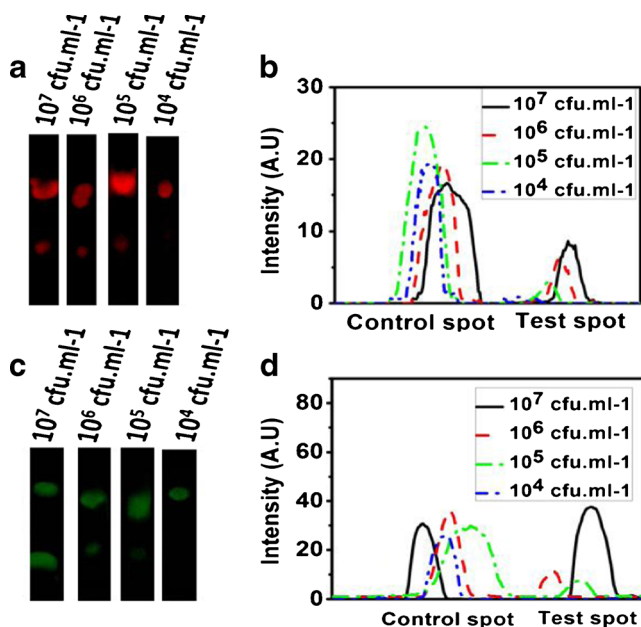
avoid the need of laptop and other computing devices for further interpretation of data. Thus, by integration of diagnostic test procedure with mobile phone an end-to-end solution for point – of – care detection in the hands of healthcare worker and clinicians has been evolved. This device can be adapted in various clinical testing applications which require rapid, bedside detection of pathogenic bacteria by simple customization of the module. These promising results form the basis of future diagnostics and create new opportunities in the examination of food, clinical and environmental samples

Conclusion

The specificity of antibodies was combined with the high signal enhancement and ease of surface functionalization offered by dye doped SiNPs to result in enhanced detection capabilities of conventional LFIA strips. Utilizing a simple smartphone based device, a reliable detection of pathogens directly in test sample at levels of 10^5 cfu.mL^{-1} has been demonstrated. This qualitative analysis of the test strip using a common smartphone may be of more utility to a clinician and affordable for routine use in developing countries.

Fig. 5 Detection of *Escherichia coli* O157 using fluorescence LFIA strip. **a** and **c)** Snapshot of image captured using smartphone based device. **b** and **d)** Intensity plot obtained using smartphone resident application

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