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Dear Editor,

We thank both the reviewers for their critical remarks and valuable comments for further improvements of the manuscript.

We have revised the manuscript with the addition of four control experiments to answer the queries raised by the reviewers. These experiments prove the high specificity of our sensor, the substantiation of the claim for high sensitivity, and the accuracy of measurements with respect to time. To make it easy for the reviewers all the changes in the document are done in red color.

We have outlined the answers of the queries raised by the reviewers below:

Reviewer 1

Q1: Lines 10~14 on Page 5of7: The working principle of the developed sensor for determining the collapse of bacterial architecture as a result of bacteriophage attack can be easily seen by SEM analysis just like it is seen in Fig. 2c-d. If the authors can visualize the intact E. coli in SEM image, it is also very much possible that the E.coli cellular deformation or collapsed structure can also be visualized by SEM. The authors need to provide this important data in their manuscript.

Answer: Though the sensor does not work on the principle of the collapse of bacterial architecture, we have added the AFM and SEM image of a sensor chip after 30 minutes of interaction (Figs. 2(c) and 2(g)), which show E coli still attached to the chip. It means that bacteria do not get lysed. Further, one additional control experiment was performed on a sensor surface developed on glass slide for a "time resolved study of the bacterial lysis" by phase contrast microscope to ensure our claim (From line 16- 59, page 7, including Figs. 8 and 9). We have cited relevant literature to emphasize our discussions (Refs. 43-46).

Q2: From the data presented in Fig. 6, the minimum level for E. coli was 150 cells. How is the authors state the detection down to a single bacterium is unclear.

Answer: Already explained. A volume of 10 microliters from a well stirred solution of 150 cfu/ml is supposed to contain the concentrations down to single bacterium (Please see lines 5-11, page 7).

Q3: Did the authors extract at different cm-1 (Raman shift) that that presented in Fig 6? Why did the large dynamic range at 1074 cm-1 in Fig. 6 and why not other regions?

Answer: Out of all the enhanced peaks, 1077 cm-1 was selected because of highest enhancement. Since, it is a kind of intensity modulated sensor, it is obvious that as compared to other peaks, the dynamic range corresponding to the peak with highest enhancement will be the largest. This has been mentioned in line 103, page 5- till line 13, page 6.

Q4: It is well known that the laser beam itself is harmful/detrimental to the intact E. coli cells, the authors need to provide information of the developed sensor tested against only E. coli in absence of bacteriophage. Who knows, the results may indeed originating from the harmful effects of laser induced damage to the cells?

Answer: In a number of reports on E coli sensing based on SERS, no one has ever observed any effect of laser on bacteria. The increase in temperature at the beam spot was experimentally measured with a thermometer and calculated analytically (using the first law of thermodynamics). Both the calculations and the experiments show an increase of less than 2 ^{0}C in temperature. Exposing E coli to a temperature 2 ^{0}C above the room temperature for 20 seconds does not make any significant effect on it.

Q5: Line 26 on Page 6of7: Cited the fig 8, the reviewer could not find fig 8 in the manuscript.

Answer: This was a typographical mistake. We have corrected it in the revised manuscript.

Q6: There are numerous types of E. coli strains existed both pathogenic and non-pathogenic. What type of E. coli did the authors used in this study is unclear.

Answer: The type of E. coli was already mentioned in the manuscript. We have added further information about other bacteria taken for the study. This has been added at relevant positions in the "Bacterial Growth and Culture" section.

Q7: For determining the specificity of the sensor, it is important to use two different strains of same E. coli species to determine the specificity of the developed sensor.

Answer: We have added one control experiment now using another strain of *E coli*. The response curves have been presented in Figs. 6 and 7 and discussed in lines 44- 58 of page 6.

Q8: The authors used minimum 150 cells and the statement "down to levels of single bacterium" is not evident from the results presented by the authors.

Answer: Already mentioned. Similar to Q2.

Q9: There are similar papers on the concept and instrumentation published by the same group. For eg., Ref. nos. 4, 12~13.

Answer: No similar papers exist on E coli sensors using nSTFs on SERS from our group. This paper does not claim a new instrumentation.

Q10: The authors have claimed single level detection of bacteria using SERS in abstract…..but as the lowest detection was achieved with 1.5X102 CFU/mL. Please revise this sentence.

Answer: Already explained. Similar to Q2 and Q8.

Q11: The novelty lies with the use of T4 phase, but it still added one more layer to the detection protocol which requires addition step for detection. This will increase the detection time as well.

Answer: The addition of T4 phage effectively does not increase the detection time, as light has to travel additional 120 nm (height of the phage) before reaching the surface. Further, addition of the phage layer makes the sensor highly specific.

Q.12: The SEM images in Fig. 2 are not clearly visible. Fig. 2 (a) needs a scale bar. In Fig. (b), as authors mentioned about the leg like structure of T4 phage is inappropriate and unclear. Fig. 1 need to be revised for better understanding of the concept with proper resolution as indexed under author guideline.

Answer: We have added new SEM images with better resolution. Further, AFM images have been presented for cross validation. (Please see Fig. 2)

Q.13: In most places authors have not italicized "E. coli" including the title!

Answer: We have italicized "E. coli" throughout the revised manuscript.

Q.14: There are numerous SERS sensors reported earlier to detect E. coli and other organisms with high specificity. Though the presented sensor utilized the use of T4 phase to capture and kill the bacteria. The present sensor is also not reusable. In this context how authors have compared the present sensor with available ones?

Answer: We have shown that T4 phage does not kill bacteria in the present case and have added proper explanation with support of suitable citations. The present sensor has many advantages such as robustness, high specificity, low limit of detection etc. Now, after the establishment of no lysis of bacteria by the phages, we have added a method to reuse these sensors. This has been mentioned in lines 72-81, page 5.

Q.15: After 30 min of incubation of E. coli with T4 phage…..the bacterium about to burst??? This statement needs to be revised with proper explanation in relation to bacteria and T4 interaction in 30 min. Also what was the evidence for selection of 30 min. Any relevant reference which support this statement?

Answer: We have extensively worked on this section and added a control experiment for studying the interaction of E coli with the phage with respect to time. Relevant literature was already cited before. Now we have decreased the interaction time to 10 minutes. (Please see lines 72-81, page 5)

Q16: What is the choice of selection of particular dilutions for experiment? How did the authors ended up starting from the minimum 150 cells? Why not testing with single cell?

Answer: The cell count of the bacteria was done by conventional plate reading (cfu) method using serial dilution and plating. The sample solutions of appropriate dilutions were made in PBS from this stock solution of the known concentration. Every time, well stirred solution was taken. We did not take single bacteria in 1 ml solution to avoid any false readings. As we already said, 10 microliter of the sample was taken from a well stirred sample solution. To ensure we got at least one bacterium in 10 microliter, we did not go below 150 cfu/ml (Please see lines 58-66, page 5).

Q17: The interpretation of data in Result and discussion need to be improvised according to the figures.

Answer: We have improved this section in the revised manuscript.

Q18: Grammatical and syntax error need a recheck, in many sentences leading to confusion.

Answer: We have rechecked the revised manuscript for possible grammatical mistakes and confusing sentences.

Q.19: The enhance response of curve of E. coli and CV026 intersect at a point. Whether we cannot predict the sample contain E. coli or not?.....until we test at least two samples. This statement is confusing.
Answer: We have resolved this issue in the revised manuscript. Please see lines 14-30, page 6 and Fig. 6.

Q.20: The lowest and highest maximum LOD and LOQ of present nanosensor?

Answer: It was mentioned in the manuscript. Please see lines 31-44, page 6.

Q21: Did authors have cross validated presented results with some other methods? What is the fate of bacteria after T4 DNA insertion?

Answer: No commercial device till date is available up to this sensitivity. So, the result cannot be cross validated with any known device. No one really cares about the fate of the sample solution once the analyte is measured. (For example no one questions what happens to blood after you measure glucose, cholesterol etc.). However, we have performed a time resolved study to check the fate of bacteria on the chip (From line 16- 59, page 7, including Figs. 8 and 9).

Reviewer2:
To answer all the queries, we have performed negative control experiments on two other bacterial strains and another *E. coli* strain. The change in the background signal is a common problem in intensity modulated sensors. That's why the response curves for both the bacteria intersected. We have resolved this issue in the revised manuscript. (Please see lines 14-31, page 6 and Fig. 6).

We have considered all the comments of both the reviewers in the revised manuscript. In light of the sincere comments from both the reviewers and our own interest, we have performed the additional experiments to strengthen our manuscript. Query-wise responses have been added in "response to reviewer's comments" file.

Though several reports on E coli detection by SERS have been reported; most of them are based on the differentiation of the E coli from other bacteria. A quantitative detection system with high sensitivity lacks in the literature. We have fabricated a novel SERS based nanobiosensor with high sensitivity, which can be used for the concentration levels of single bacterium. In this way we have miniaturized the sensor size and enhanced the sensitivity and demonstrated the specificity of our sensor.

We have considered all the comments of the reviewers in the revised manuscript. In light of the sincere comments from reviewer 2 and our own interest, we have performed the additional experiments to strengthen our manuscript.

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PAPER

Highly sensitive and specific detection of *E. coli* by a SERS nanobiosensor chip utilizing metallic nanosculptured thin films

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A nano-biosensor chip, utilizing surface enhanced Raman spectroscopy (SERS) on nanosculptured thin films (nSTFs) of silver, was shown to detect *Escherichia coli* (*E. coli*) bacteria down to a the concentration levels of a single bacterium. The sensor utilizes highly enhanced plasmonic nSTFs of silver on a silicon platform for the enhancement of Raman bands as checked with adsorbed 4-Aminothiophenol molecules. T-4 bacteriophages were immobilized on the aforementioned surface of the chip for the specific capture of the target *E. coli* bacteria. To demonstrate that no significant non-specific immobilization of other bacteria occurs, three different, additional bacterial strains, *Chromobacterium violaceum*, *Paracoccus dentrificans* and *Pseudomonas aeruginosa* were used. Furthermore, experiments performed on an additional strain of *E. coli* to address the specificity and reusability of the sensor showed that the sensor operates for different strains of *E. coli* and is reusable. Time resolved phase contrast microscopy of the *E. coli*-T4 bacteriophage chip was performed to study its interaction with bacteria over time. Results showed that the present sensor performs a fast, accurate and stable detection of *E. coli* with ultra-small concentrations of bacteria down to levels of a single bacterium in 10 μl volume of the sample.

Introduction

Biosensing using Surface enhanced Raman spectroscopy (SERS) has emerged as a popular field of research since the past decade due to its putative low detection limit, high sensitivity and specificity as well as other intrinsic exciting properties. A number of biosensors based on SERS for the detection of glucose, cocaine, DNA, endocrine disruption compounds (EDCs), *E. coli*, etc. have been reported[1-5]. SERS is the phenomenon of enhancement of the Raman spectroscopy signal of certain molecules by a factor of several orders of magnitude when they are brought in contact with nanostructured metal surfaces. This enhancement in intensity is attributed to the highly localized fields of plasmons in the vicinity of the molecule emitting Raman signals, and many theoretical and experimental studies have been performed to elucidate its dependence on various factors[6-7] (shape, size, orientation, porosity, material of the nanostructure and the substrates on which the nanostructures are deposited).

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In order to modulate signal enhancement a number of structures were studied, including nanorods, nanowires, nanocubes, dielectric-metallic core-shells, nanoflowers, nanosculptured thin films (nSTFs) and many other shapes of different material compositions and these were examined for SERS enhancement[8-12]. nSTFs have unique properties such as large enhancement factor, stability, reproducibility, durability, ease of fabrication, large scale surfaces, cost effectiveness, etc. nSTFs are nanorod like structures with different surface morphologies which are generally grown by the glancing angle deposition technique (GLAD)[13]. Previous studies in our group established the optimization of the performance of the SERS enhancement of nSTFs with respect to their material composition, height, underlying substrates and porosity[4, 12] and those determined to be optimal were used in the present study.

Escherichia coli is a gram-negative, facultative anaerobic, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. *E. coli* is considered to be an indicator for fecal contamination (fecal coliforms) and some strains are pathogenic, responsible for food or water borne, gastrointestinal diseases. Consumption of water and/or food contaminated with *E. coli* can lead to hemolytic-uremic syndrome (HUS), especially in children and elderly people. HUS causes the destruction of red blood cells and kidney failure, which may lead to stroke, seizures, and even death[14]. According to surveys of the World Health Organization (WHO), approximately two billion people get affected by gastrointestinal diseases annually[5].

According to the statistics of the United States Centres for Disease Control and Prevention (CDC), about 95,000 people get affected annually by *E. coli* in the US alone[15]. Therefore, *E. coli* has become a prime target for detection and cure. A number of classical methods (plate counting, polymerase chain reaction (PCR), ELISA), as well as, state-of-the-art methodologies including physical transduction methods such as surface plasmon resonance (SPR), long period fiber gratings (LPG), surface acoustic waves, surface enhanced fluorescence (SEF), microelectronic mechanical systems (MEMS), amperometric detection, microfluidics integrated micorscopy, surface enhanced Raman spectroscopy (SERS), fiber optic immunosensors, etc have been reported for the detection of *E. coli*[16-26]. SERS has been found to provide the lowest limits of detection from orders of magnitude in its enancement[27]. Since the Raman bands of a chemical/biochemical species are unique, the enhanced Raman is considered one of the most accurate methods for detection. However, since the Raman enhancement is a very short range phenomenon, the complete Raman bands of only very small species can be assessed by SERS[4]. The complete information of all the Raman bands from bigger species such as bacteria is thus inaccessible. Most of studies based on SERS based detection of *E. coli* bacteria are based on spectral differentiation at high concentrations and are susceptible to interferences from the culture medium[28]. There is still only limited literature available which discusses the sensitivity of such sensors. Such a study was recently reported with nSTFs on filter papers [28]. However, the limit of detection of these sensors remains quite high. Furthermore, specificity of a sensor requires the use of affinity-based capture recognition elements such as antibodies, Nucleic acids (DNA/RNA), Aptamers, bacteriophages, glycoproteins, etc.[5, 21, 29-32]. Antibody-based sensors suffer from the possibility of cross-linking to unrelated bacteria exhibiting similar molecular structures, thus, resulting in false signals[33]. Aptamer based sensors are particularly suited for hapten-sized target molecules but it is still of limited use in large entity capture such as that of bacteria. In addition, neither antibodies, nor aptamers enable the discrimination between viable and non-viable cells[34]. Bacteriophage-based sensors exhibit high specificity (for their host bacteria) and relatively better stability than antibodies (from heat, alkali and acidic solvents)[32]. However, unlike antibodies and aptamers they need to be isolated from nature, however, once this is done they are easily cultured. Their specificity can be modulated in the laboratory through complicated laboratory-based mutation studies. Apart from that, though bacteriophages can easily discriminate living bacteria as they do not infect dead cells, and this by injecting their DNA thus prompting the production of phages and subsequent lysis of the host cells[22]. However, the sensor can be reused if there has been no specific recognition and therefore the phages are still intact[23].

In the present study, we have fabricated chips of silver nSTFs over a Si substrate for the development of the sensor. SERS signals from model 4-aminothiophenol (4-ATP) were assessed for their sensing power. Specificity of the sensor was given by functionalization with T4 bacteriophages as the capture biomolecular recognition element. SERS spectra were recorded for two different strains (*E. coli B* and *E. coli μX*) at different concentrations, as well as, three unrelated control bacteria, namely *Chromobacterium violaceum*, *Paracoccus dentrificans* and *Pseudomonas aeruginosa* to confirm the fact that our sensor does not suffer from non-specific binding to the surface. Another control experiment was performed to observe the interaction/possibilities of lysis of *E. coli* on the sensor surface by developing the sensor protocol on a glass micro-slide. The binding/interaction of the attached phage - *E. coli* was recorded by a phase contrast microscope at different time intervals.

Materials and methods

4-Aminothiophenol (422967), glutaraldehyde (G7651),, and bovine serum albumin (BSA) (A2153), (3-Aminopropyl) trimethoxysilane (281778) (aminosilane) were purchased from Sigma. Distilled water of 18 MΩ-cm resistivity was obtained from a Millipore® system. Phosphate buffer saline (PBS) was obtained from Dulbecco. Sulphuric acid (H2SO4, 98% pure) (19550501), hydrogen peroxide (H_2O_2, 30%) (08550323), ethanol (C_2H_5OH, dehydrated, 99.9% pure) (05250502), and acetic acid (CH_3COOH, 99.8% pure) (01070521), were purchased from Bio-Lab Ltd., Israel. All the chemicals were used as obtained without further purification.

Bacterial Growth and Culture

Frozen stocks of bacterial strains *E. coli* RFM443[35] (*E. coli B*, ATCC® 11303™), *E. coli XLMRF* (*E. coli μX*), *Paracoccus dentrificans* (*P. dentrificans*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Chromobacterium violaceum 026* (*C. violaceum*)., a gift from Prof. P. Williams (University of Nottingham, Nottingham, UK), were used as seeds for cultivation in 10 ml LB (Difco Luria–Bertani medium, BD, France) grown overnight at 37°C and 30°C respectively in a rotary thermo-shaker (Gerhardt, Germany) at 120 rpm. The overnight culture was centrifuged for 10 min at 3000×g, the pellet was washed and re-suspended in 0.01 M phosphate-buffered saline (PBS), pH 7.2. The bacterial concentrations were determined using the colony forming units (CFU) method. *C. violaceum* is also a gram negative bacterium, but evolutionary distant enough from *E. coli*, and cannot be recognized by T4 bacteriophage. Similar to *E. coli*, *C. violaceum* is commonly found in soil and aquatic environment, but is not present as a part of the normal flora of humans and animals[36-37]. *P. aeruginosa*, an opportunistic pathogen, is also found in water, soil, skin flora and man-made environments and is commonly involved in drinking water contamination events, while *P. dentrificans* is a soil bacterium[38-40]. They both are gram negative bacteria.

T4 bacteriophage preparation

Phage propagation was done as described before[23]. A T4 phage culture was incubated with an *E. coli* broth, then added to fresh LB media and incubated as described before for 6 hours. The culture was centrifuged and the supernatant filtered. The phage suspension was centrifuged inside an Amicon® Ultra-0.5 (MILLIPORE) and the resulting phage pellet resuspended in SM buffer[41]. Using the soft agar overlay technique the phage was enumerated by plaque forming unit (PFU).

n-STF Fabrication

Nanosculptured thin films of silver were prepared by glancing

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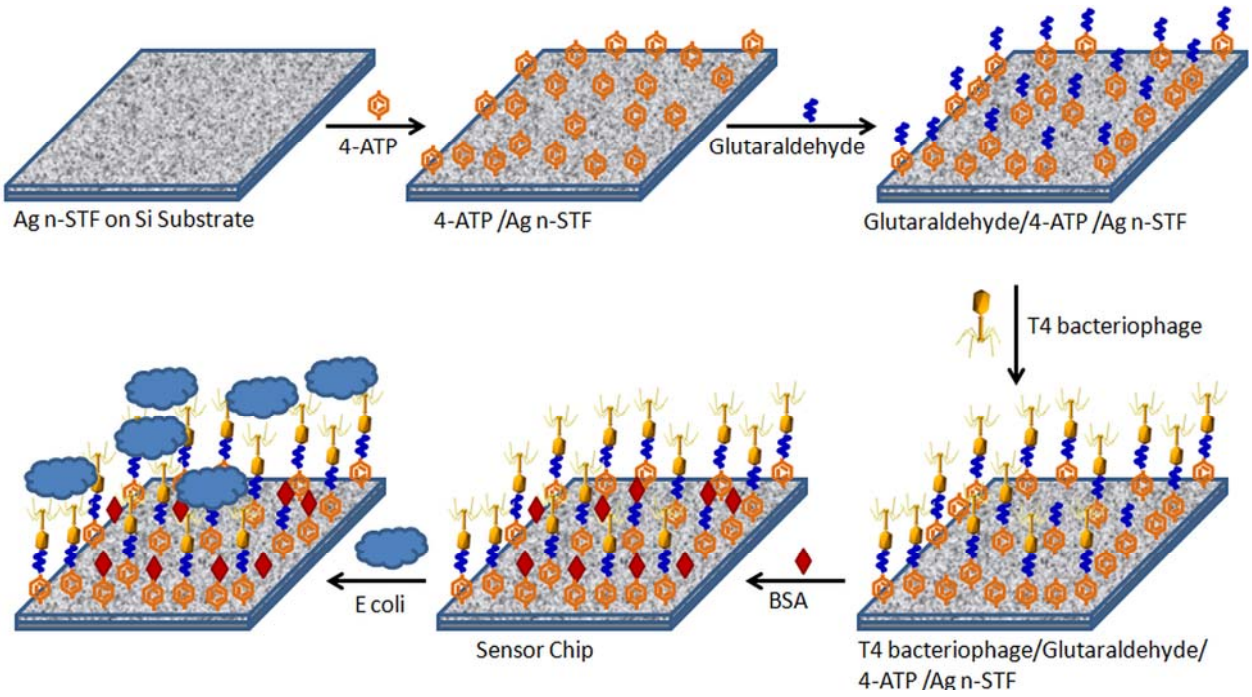

Fig.1 Schematic of step by step sensor chip development

angle deposition technique (GLAD) described elsewhere[13]. Briefly, the Si substrate was kept in a vacuum chamber at an angle called glancing angle to the incident metal plume. The various parameters of the coated film, such as thickness, porosity, topography were controlled by adjusting suitable temperature, pressure, tilt angle, rotation speed etc. It has already been reported that Ag n-STFs having about 300 nm in height and 30% porosity over silicon substrates possess the highest SERS enhancement[4, 12-13]. Therefore, the n-STFs with optimum performance were fabricated for the present study.

Sensor Chip Development

A small piece (about 5-7 mm^2) of the fabricated n-STF was incubated overnight in 4-ATP solution in ethanol (1% w/w). As a result, a self-assembled monolayer of 4-ATP was spontaneously allowed to form over the Ag surface. The chip was then taken out of the 4-ATP solution and rigorously washed with ethanol and a continuous flow of water to remove any remnants. The chip was dried by blowing nitrogen gas. The chip was then incubated in aqueous solution of glutaraldehyde (5% v/v) for 1 hour to form a cross-linking layer. After taking the chip out of the glutaraldehyde solution, it was again washed with water to remove any unbound molecules and dried with a blow of nitrogen gas. The chips were then further incubated in T4 bacteriophage solution for 4 hours to form the specific receptor layer for the target *E. coli B* bacteria. Thereafter, the chip was incubated in BSA solution of 1 mg/ml concentration in 50mM PBS buffer for 1 hour to block any remaining empty surface sites to prevent putative non-specific binding on the sensor surface. This step leads to increase in specificity and hence the performance of the sensor, while reducing the effect of interference from other substances present in the sample which may add falsely to the signal. After BSA incubation, the chip was taken out, washed rigorously in water and PBS and blow dried with nitrogen. The chip was stored in a refrigerator at 4°C prior to characterization. A schematic of the stepwise sensor chip fabrication is shown in fig.1.

The Atomic force microscope (AFM) and Scanning electron microscope (SEM) images of the sensor chip before and after the surface functionalization are shown in figs. 2(a), (b) and (d), (e), respectively. In both the AFM (2(b)) and the SEM (2(e)) images, the leg like structures of the immobilized T4 bacteriophage are clearly visible at the sensor surface after functionalization, while the n-STF surface is also visible in the background of the functionalized material. Figures 2(e) and (f) show the SEM image of a number of *E. coli* attached to the sensor surface, while figs. 2(g) and (h) show high resolution images of a single bacterium attached to the sensor surface. This clearly indicates that a single bacterium is attached to multiple phages immobilized on the sensor chip. On average, the sensor chips were 5-7 mm^2 of size. Since the area of the focussed spot of the laser beam (in fig. 3) was about 90 μm^2, even smaller sensor chips up to dimensions of 100 μm^2 will be sufficient. However, the greater dimensions were used due to the ease of handling. The images 2(c) and (g) were taken after 30 minutes of interaction to see whether the bacteria became lysed with time or not.

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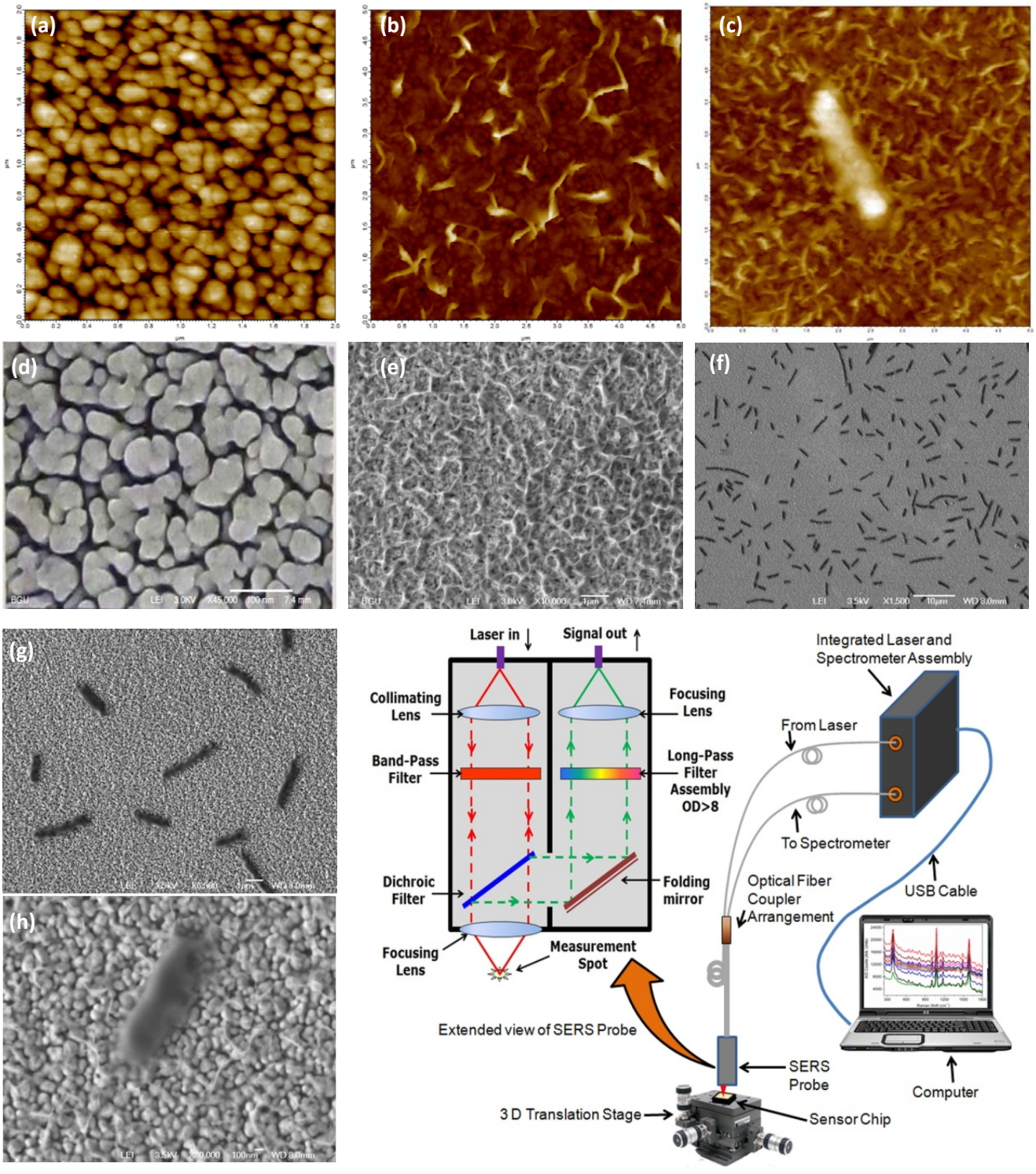

Fig.2. AFM and SEM images of the nSTF sensor: (a), (d) before and (b), (e) after functionalization, respectively; (f), (g) SEM images of *E. coli B* attached to the sensor surface, and (c), (h) High resolution AFM and SEM images (respectively) of a single *E. coli B* attached to the sensor surface

Fig.3. Schematic of the experimental setup

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Experimental Setup

An illustration of the experimental setup is shown in Fig. 3. A fiber optic Raman spectrometer was used in these studies. It consists of both a laser and a spectrometer assembly housed in a single package. The light from a 785 nm laser was coupled to the proximal input end of a fiber optic coupler arrangement and the collected signal light from the far end of the coupler is fed to the spectrometer. Light from the far end output of the fiber optic SERS probe was focussed on the desired spot on the sensor chip with the help of a three dimensional (3D) translation stage. The captured enhancement is found maximum at the focus. The Stokes lines were captured from the same aperture and sent to the spectrometer via another optical fiber and suitable optics. The spectrometer was interfaced to a computer which further translated the captured signal into the SERS spectrum. An extended view of the SERS probe at the end of the optical fiber coupler is also shown in the same figure. As is evident, light from the laser is first collimated and then passed through a band pass filter to ensure the excitation at a single wavelength range and focussed on the sample by another lens. The enhanced Raman signals as well as the reflected light from the laser, are captured by the same lens. The Raman signals are filtered from the reflected light by a dichroic filter and sent to the collecting assembly. The light from the dichroic filter is sent to a long pass filter assembly by a folding mirror. The long pass filter again filters the Rayleigh scattered light from the Raman scattered wavelengths. The Raman scattered light transmitted through the long pass filter assembly is then focussed on the input end of the collecting optical fiber by another lens. The operation principle of the sensor is that a change in the SERS enhancement of the sensor chip occurs when the bacteria are captured by the bacteriophage and thus brought intimately to the sensitive surface.

As the SERS is a very short range phenomenon, we assessed only the SERS spectra from 4-ATP adsorbed on the surface of the n-STF. There is almost negligible change in the SERS spectra due to further binding of glutaraldehyde, bacteriophage and BSA. It has already been established that further binding does not affect much the Raman bands of the molecule nearest to the metal surface.[4] Further binding of molecules may only result either in increase or decrease in the Raman enhancement. The change in enhancement can further be translated into the concentration of the binding analyte molecule. This is the working principle of such sensors. When the bacterium binds to the bacteriophage, it inserts its DNA into the bacterium which further replicates very quickly into the bacteria. After a certain amount of time (30 min), the bacterium is full of bacteriophages, about to burst. Certain sensors utilizing refractive index change operate on this principle so that the response could be recorded around the peak time, when the bacterial matrix is modified the maximum.[22] However, it was also shown that if the interaction is carried out only up to the phage- E. *coli* association time, the mechanism can be used in SPR based detection methods[23]. Such sensor surfaces could be regenerated for further use. In the present case, the bacterial association on the sensor surface was carried out for 10 minutes and the surface was regenerated for further use. The integration time of the spectrometer was 20 seconds.

The sample solutions of different concentrations ranging from 150-10^5 cfu/ml were prepared in PBS buffer from the stock solutions of *E. coli B*, *P. aeruginosa* and *C. violaceum* bacteria by making appropriate dilutions from stock solutions of $1.5X10^8$, $3X10^7$ and $1.5X10^9$ cfu/ml concentrations, respectively. Similarly, the sample solutions of *E. coli μX* ranging from 340-10^5 cfu/ml and *P. dentrificans* ranging from 265-10^5 cfu/ml were prepared from the stock solutions of $3.4X10^8$ and $5.3X10^8$ cfu/ml concentrations respectively. The sensor chips were incubated in the sample solutions for 10 minutes and then taken out and washed with PBS buffer twice. The remaining moisture/liquid was taken out by lightly and carefully blowing with air. The excess liquid was taken out to avoid any lensing effects due to the droplet on the sensor chip. The SERS spectra were then recorded from at least three places on the same sensor chip. After the recording of the spectra, the sensor chip was washed in running NaOH aqueous solution (20 mM) for three minutes. This washing helps remove the attached bacteria from the surface and hence regenerate the sensor surface[23]. The sensor surface was then rinsed twice with PBS to remove the remnants of the NaOH solution. The chip was blow dried again with air and incubated in other sample solution. This procedure was repeated for all the sample solutions at different concentrations of all kinds of bacteria used in the present study. The SERS spectra recorded from the three spots at the chip were averaged.

Results and discussion

The recorded SERS spectra for different concentrations of *E. coli B* are shown in fig. 4. It is observed that there is a change in the SERS enhancements at different concentrations but no trend of variation can be predicted. However, when all the SERS spectra are referenced to the zero level, a clear trend in the variation of the enhancement with increase in the bacteria concentration is observed. As can be seen in the inset, where we have plotted the Raman enhancement of the 1077 cm^{-1} band for different concentrations of *E. coli B*, the enhancement increases with an increase in the bacteria concentration and becomes almost saturated. Similar SERS spectra were recorded for the varying concentrations of *E. coli μX*, *P. dentrificans*, *P. aeruginosa* and *C. violaceum* bacteria. The SERS spectra for *CV026* have been plotted in fig. 5. It is easily evident that almost no change in the SERS enhancement was observed, even from the inset, which was similar to that for *E. coli B*, where we have plotted the Raman band at 1077 cm^{-1} for different bacterial concentrations.

To make a more quantitative prediction of the response of the sensor, we plotted the Raman enhancement versus concentration at 1077 cm^{-1} for all kinds of bacteria in fig. 6. The Raman peak at

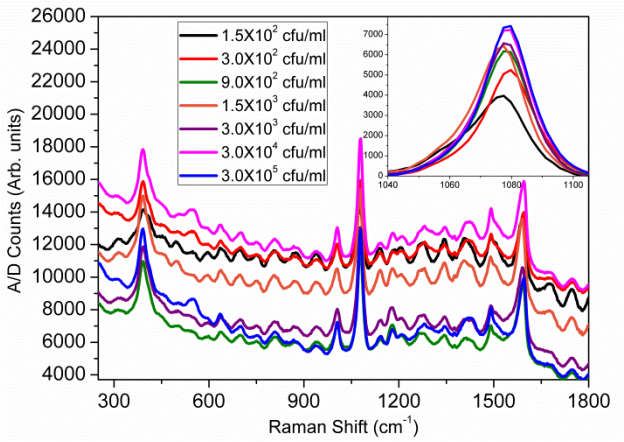

Fig.4. SERS spectra for varying concentrations of *E. coli B*. The inset shows the variation of Raman enhancement with concentration at 1077 cm^{-1}

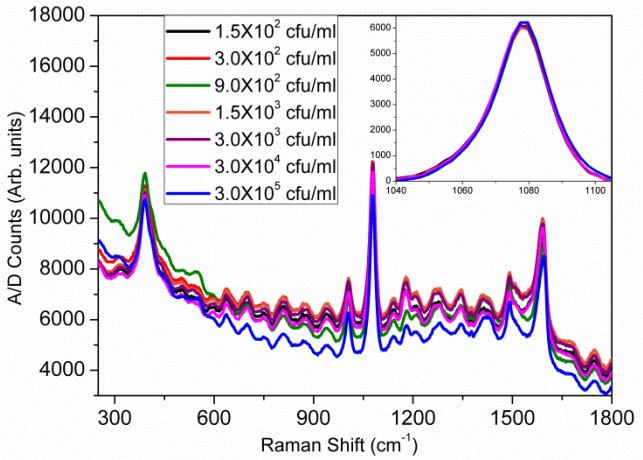

Fig.5. SERS spectra for varying concentrations of *C. violaceum*. The inset shows the variation of Raman enhancement with concentration at 1077 cm^{-1}

1077 cm^{-1} was selected because of the maximum enhancement at this band, which corresponds to the largest sensitivity and dynamic range of the sensor. The concentration at the x-axis is logarithmic due to very large dynamic range of the sensor. The background Raman enhancement from the bare sensor chip was subtracted from the SERS signal of samples over the sensor to avoid any unavoidable deviations due to fluctuations in the background. Hence, the Y-axis represents the A/D counts from the sample minus that from the sensor (I_{Sample} - $I_{Background}$) at 1077 cm^{-1}. Throughout the manuscript we designate the processed signal as the differential Raman enhancement. The symbols represent the differential Raman enhancement at different concentrations of different bacteria extracted from the respective SERS spectra, while the lines through them are the best curve fits. It is clear that with an increase in the concentration of both types of *E. coli*, the differential Raman enhancement first increases and then becomes nearly constant. However, for *C. violaceum*, *P. dentrificans* and *P. aeruginosa* there is almost no change in the differential Raman enhancement with an increase in the concentration. This confirms the specificity of the sensor for *E. coli B* detection. The successive addition of bacteria on the sensor surface contributes a little to the Raman enhancement,

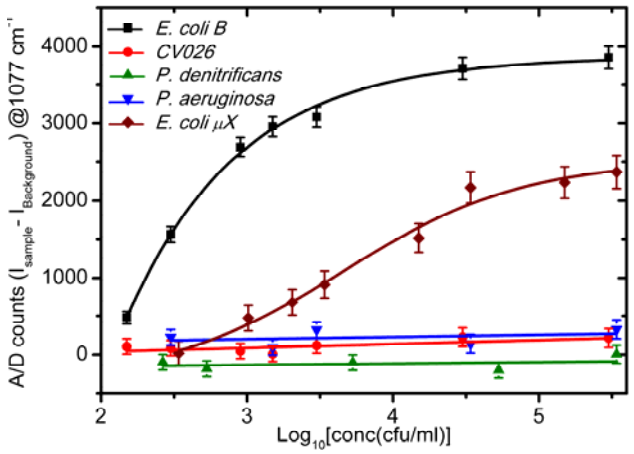

Fig.6. Response curve of the sensor for various bacteria

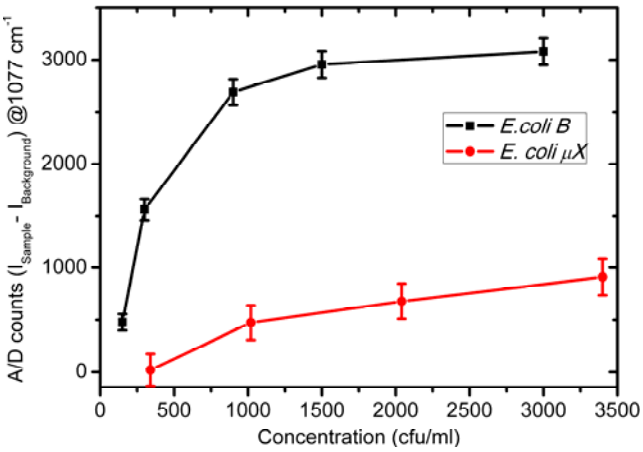

Fig.7. Sensor response to *E. coli* bacteria at smaller concentrations

which leads to an increase in the signal. The region of saturation of the sensor response is less sensitive and hence puts a higher limit on the range of bacterial concentration, which can be detected by the sensor. This is due to the small size of the focussed laser beam, which limits the number of observed bacteria binding to the active region (exposed to the laser) of sensor surface. Furthermore, it was observed that the sensitivity of the sensor is greater for *E. coli B* than that for *E. coli μX*. It may due to a lower affinity of *E. coli μX* strain to T4 bacteriophage than E. coli B. Thus this possible lower affinity of *E. coli μX* may lead to reduced binding at the surface and hence to lower sensitivity. However, due to the reduced sensitivity, while the sensing spot remaining the same size (= the spot size of the laser beam, 90 μm^2) the dynamic range of the sensor becomes larger for *E. coli μX*. Furthermore, this sensor can detect whether an unknown sample contains any *E. coli* or not in the first instance, but an additional set of measurements are required to distinguish between different *E. coli* strains. To estimate the exact cell count and the *E. coli* strain, one should make further dilutions (at least one) of the unknown sample to see the matching of the response curve to that of one of the two *E. coli* bacterial strains. This provides an accurate measure of whether the sample solution contains *E. coli* or not and at what concentrations. We plotted the response of the sensor for very low bacterial concentrations varying from 150 to 3000 cfu/ml in fig. 7. The error bars in figs. 6 and 7 were calculated by taking into consideration the least

count of the pipettes, and the noise level of the SERS measurement. It is quite clear from the plot (fig 7) that *E. coli B* concentrations down to 150 cfu/*ml* (~ 1 bacterium per 10*μl*) and *E. coli μX* concentrations down to 340 cfu/ml can be detected with the present sensor. The sample volumes in our experiments were kept at approximately 10 microliters, which means that such a volume when taken from a well-mixed sample of 150 cfu/*ml* will nearly always have at least a single bacterium in it. In this way, we can say that the sensor is capable to detect bacterial concentrations down to a single cell level in 10 μl volume of the sample. The Raman spectra for the sample solution concentrations below 150 cfu/ml were not analyzed to avoid any inconsistencies in the SERS signals, which may occur due to sometimes missing of even a single bacterium in the sample volume on the sensor surface.

Since, in general, the interaction between a T4 phage and *E. coli* results in the lysis of the bacteria, it might be possible to affect the signal of the sensor, if not properly monitored in time. To learn the fate of the bacteria on the sensor surface and understand the interaction with respect to time, we fabricated the sensor surface on glass micro-slides using a protocol similar to Tripathi et al[22]. Briefly, the glass slides were treated in piranha solution (3:1, H_2SO_4: H_2O_2) for five minutes, then rigorously washed with running DI water and finally blow dried in a nitrogen stream. Furthermore, a thin aminosilane layer was created over the glass slides by incubating them in a 1% (v/v) solution of trimethoxy aminosilane in (10:4) C_2H_5OH + CH_3COOH solution. The acetic acid prevents the formation of multilayers of aminosilane[42]. The glass slides were further incubated in 1% (v/v) aqueous solution of glutaraldehyde and then in the bacteriophage solution, as described earlier in the "Sensor chip development" section. The *E. coli B* suspension in PBS was poured on the micro-slide chip for 10 minutes and then washed twice by PBS to remove any uncaptured *E. coli*. Further the sensor surface of the chip was immersed in PBS with the help of a 70 micron spacer and a cover slide. A schematic of the microscopic slide arrangement, which was used for time resolved phase contrast microscopy, is shown in fig.8. The captured images from the phase contrast microscope at different time intervals, over a period of 1 hour are shown in fig. 9. It is quite evident that there is no change in bacterial count over time, indicating that *E coli* cells are not lysed by the bacteriophages. Had the *E coli* cells been lysed, the number of bacteria observed in the phase contrast microscope would have decreased. This result was supported by various reports already present in the literature[43-46]. The reason behind this kind of observation is attributed to the fact that bacteria are lysed by the bacteriophage only in the growth culture media and when they are free floating. In the present case, since the T4 bacteriophages are immobilized and the bacteria are in PBS, a non- growth medium, no lysis happens. It means that the results should not get affected with respect to time. Absorption of the phages to the surface may change the plasticity and the movement of the phage and prevent phage DNA injection. In order to inject DNA/RNA the phages need to exhibit conformational changes and contraction[43-44]. Bacteriophages with contractile tails epitomize the concepts of "virus" and "phage" for many because the tails of these phages undergo a large conformational change – resembling the action of a syringe – upon the attachment to the host cell.

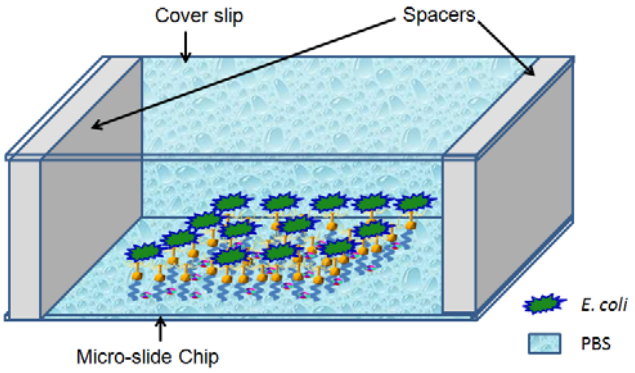

Fig. 8. Schematic of the micro-slide chip for phase contrast microscopy (components are not to scale)

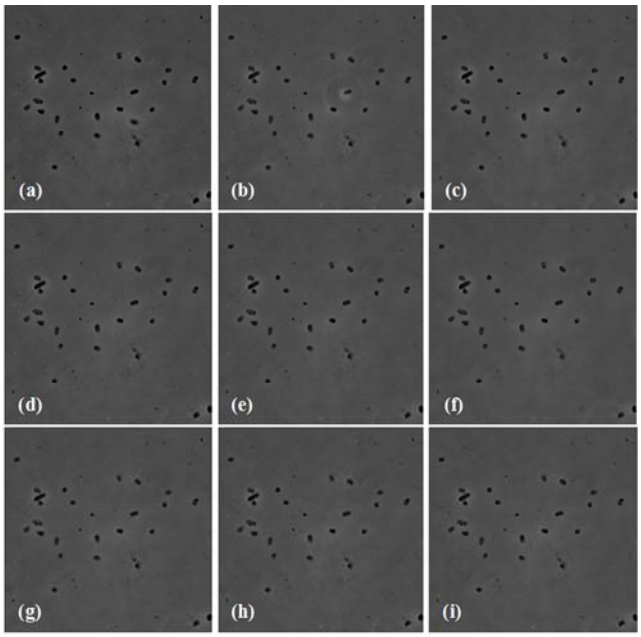

Fig. 9. Time resolved Phase contrast microscopy images of the immobilized T4 bacteriophage- E coli interaction in PBS: (a) 20, (b) 25, (c) 30, (d) 35, (e) 40, (f) 45, (g) 50, (h) 55 and (i) 60 minutes

Since the spot of the laser beam was about 90 microns, multiple micro-sized spots of the same sensor chip can be used for the detection of multiple samples. The nSTFs are very cost effective, as large, uniform chips can easily be fabricated. Additionally, the sensor chip is reusable, which makes it more cost effective. Other than that, the integration of this sensor chip with an optical fiber makes it capable of remote sensing and field applications. Furthermore, the sensor is shown herein to be faster and more sensitive than many commercially available *E. coli* detection kits.[14]

Conclusions

We have fabricated a nanobiosensor chip for the specific and quantitative detection of two different strains of *E. coli*. The sensor utilizes SERS based detection over optimized nSTFs of Ag over Si. Control experiments with *C. violaceum*, *P. dentrificans* and *P. aeruginosa* confirmed the lack of non-specificity of the sensor. The time resolved phase contrast microscopy confirmed that no lysis of bacteria happen on the

sensor chip, which confirm our measurements are time independent. The sensor could detect *E. coli B* concentrations down to 1.5X10^2 cfu/ml, which is an order of magnitude lower than a recently reported SERS sensor[28]. The sensor utilizes low volumes (10 µl) of the sample solution. In addition, it was shown to be capable of detecting *E. coli B* concentrations down to a single bacterium. One should note that phages can be stored for a long time on a surface, making the system amenable to long-term storage as a product.

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