Immobilizing Siderophores on Solid Surfaces for Bacterial Detection

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The article reports a proof of concept validation of bacterial detection using Siderophores. Desferrioxamine B (DesfB) was used as the Siderophore to capture Escherichia coli on gold coated microcantilever surface. Self-assembled monolayer based gold thiol chemistry was used for surface functionalization of the Siderophore on top surface of the microcantilever. The bacterial attachment to the Siderophore was observed through Fourier Transform Infrared Spectroscopy (FTIR) and Fluorescence Microscopy. The DesfB coated surface allows only the live bacterial cells to be attached and it is evident through the FTIR band formation. In a mixture of live and dead E. coli cells, the Fluorescence Microscope image indicates green emission from live cells and a core-shell structure formation upon progress of time. For a sample dilution of 10−1, the mass change of live E. coli bonded to Siderophores is four times higher than that of dead cells and 12 times higher to that of negative control on microcantilevers. Therefore this study should be considered as a foundation to build a miniaturized biosensing platform to distinguish between bacterial or viral infections in real time. The proposed platform could differentiate between bacterial and viral infections thus rendering it as Point of Care (PoC) diagnostic tool aiding Internet of Things (IoT) applications.

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The ability of microcantilever sensors to perform relatively fast, easy, small volume analyte requirement, and label-free analysis of biological species justifies the growing interest of the biosciences community in these devices.11 The microcantilever sensors have been successfully applied in studies on single cell estimation,12 modeling of drug action mechanisms,13 cancer research,14 and detection of pathogens and nerve agents.15,16 In this study, the authors envision to demonstrate the sensing of bacterial viability through microcantilevers enabled by SDPs. Furthermore, the focus of this work to show that it is possible to implement SDP based differentiation of live bacteria from dead cell by resonant frequency change of the microcantilevers due to mass loading rather than being aimed at detecting very low concentrations (such as picomolar) of bacteria or to selectively differentiate certain bacteria from other microorganisms.

In this research, Desferrioxamine B mesylate salt (DesfB), a bacterial SDP characteristic of Streptomyces species, is used to detect live E. coli and differentiate it from dead bacterial cells. The binding of DesfB to the E. coli SDP receptor, FhuD, is well documented, including the reported crystal structure of DesfB bound to FhuD.17 Mass loading on the microcantilever is taken as the sensor signal. The mass loading is recorded after each surface treatment of the microcantilever.

Materials and Methods

DesfB functionalization.—P-type silicon wafer (100) and microcantilever arrays (IBM, Zurich, Switzerland) consisting of eight identical cantilevers of dimension 750 × 100 × 1 μm3 were used as samples. Due to the fact that all eight cantilever beams were attached in a single array, functionalization of all beams were done together and
it assumed that all beams were functionalized uniformly. The first step of the functionalization process is to deposit metal on the cantilever. 5 nm chromium layer and 20 nm gold layer were deposited sequentially on both the wafer and microcantilever sample surface through E-beam evaporation system (model no: BC-300T). The silicon wafer was diced into 1 cm x 1 cm size samples by ADT dining instrument. Then, individual Si wafer samples (1 cm²) and the microcantilevers were cleaned with acetone, isopropanol alcohol and finally rinsed with ethanol and deionized water (DI water) separately. Next, the samples were submerged in ethanol solutions containing 1 mM of 11-mercaptopropanethiol undeconoic acid to form self-assembled monolayer (SAM) for 12 hr. After the exposure period, the individual samples were washed in hot ethanol and DI water environment.

To covalently immobilize the DesfB to the SAM adsorbed on the gold surface, the SAM terminated surface was activated with a 1:1 volume mixture solution of 0.1 M N-hydroxysuccinimide (NHS) and HCl stabilized 0.4 M 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) for 1 hr at room temperature and finally washed with DI water. Each sample was immersed in a solution containing DesfB, sodium carbonate, and FeCl₃ (1 mM each) for 2 hr at room temperature and then subsequently rinsed with DI water. This iron chelated DesfB immobilization strategy utilizes the Siderophores’ terminal amine, which is not critical to iron bonding.

Preparation of live and dead bacterial suspension: attachment on wafer.—E. coli was grown overnight in Luria Bertani media at 37°C. For quantitative determination of bacterial population, a standard agar plate count method was performed with serially diluted bacterial stock solution. A 1:1 volume mixture solution of 0.1 M N-hydroxysuccinimide (NHS) and HCl stabilized 0.4 M 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) for 1 hr at room temperature and finally washed with DI water. Each sample was immersed in a solution containing DesfB, sodium carbonate, and FeCl₃ (1 mM each) for 2 hr at room temperature and then subsequently rinsed with DI water. This iron chelated DesfB immobilization strategy utilizes the Siderophores’ terminal amine, which is not critical to iron bonding.

To identify if the bacterial strain used in this experiment scavenges the earlier published work. In the context of surface functionalization, 11-MUA molecules bind to the Au surface through the S–Au bond at one end while the carboxyl group is free at the other end to form a microtube and mixed thoroughly before use. 3 μL of mixed dye mixture was added for every 1 mL of PBS buffer (pH 7.4). Exclusively, 20μL of the dye mixture was coated on individual wafer sample which were already mounted by both positive and negative control for 15 minutes under dark ambiance. Excess amount of unbound dyes were washed by PBS buffer twice.

Mass loading on microcantilever.—Experiments were performed in Cantisens CSR-801 system supplied by Concentris Inc. All measurements were done in DI water environment. The sample chamber has a volume of 5 μL and the temperature set in the chamber at 25°C. DesfB functionalized microcantilever was inserted inside the chamber and the samples to be measured (here live E. coli, dead E. coli and Human IgG) were injected separately into the chamber. An automated pump was programmed to pick up the sample solution at a constant speed of 4 μL/s. DesfB functionalized microcantilevers interact with the sample solution and the resultant resonance frequency shift (Δf) of the microcantilever recorded infers additional mass loaded on microcantilever due to DesfB and E. coli interaction. For the given dimension of microcantilever, the 19th mode was selected as the experimental resonant mode for its high signal to noise ratio. Upon target binding, cantilever will deflect if operating in static mode or its resonant frequency will shift if operating in dynamic mode. In this present study continuous tracking of the resonant mode was performed with Phase Locked Loop (PLL) due to its higher sensitivity upon mass loading. Integral and Proportional gains of the PLL were adjusted to achieve real time accurate tracking of the resonant mode with minimum noise in signal.

Results and Discussion

ATR-FTIR spectra of attached DesfB on functionalized surface.—FTIR analysis of 11-MUA SAM and surface functionalization with NHS/EDC chemistry in this study correlates well with the earlier published work. In the context of surface functionalization, 11-MUA molecules bind to the Au surface through the S–Au bond at one end while the carboxyl group is free at the other end to react with the NHS/EDC. The intermediate NHS/EDC ester binds the iron chelated DesfB with the self assembled monolayer of 11-MUA covalently. The DesfB molecule consists of three bidentatehydroxamic acid groups to form a hexadentate ligand. The residual chain includes two secondary amide groups and an aliphatic chain. Upon iron chelation, the oxygen atoms of the hydroxamate groups bind to the iron molecule with deprotonation of three hydroxyls to form a three identical asymmetrical chelation rings, Ferrooxamine B molecule. The open chain, ending with a saturated amine group on one edge of the linear molecule gives the molecule a positive charge. A comparison of the FTIR spectra of DesfB and its iron chelated complex Ferroxamine B is presented in Fig. I and their consolidate wave numbers are tabulated in Table I.
After SAM formation and prior to DesfB coating, the DesfB molecule was treated in a solution containing sodium carbonate and FeCl₃ (1 mM each) to form its iron complex, Ferrioxamine B. The broad band at 3317 cm⁻¹ is assigned to the N-H of the secondary amide (Table I). Iron chelated DesfB has the secondary amide shifted from 3317 cm⁻¹ to 3287 cm⁻¹ corresponding to bond formation.

An intensive overlapping of vibrations was also exhibited due to the iron chelation seen in case of bonding with the -COOH group of 11-MUA. Furthermore, a single band at 3087 cm⁻¹ assigned to C-N-H overtone appears in presence of two individual bands. During Ferrioxamine B formation, the band assigned to CH₃ at 2960 cm⁻¹ shifts to lower wavenumber at 2950 cm⁻¹. The asymmetric CH₂ band is further shifted to the lower wavenumber at 2926 cm⁻¹ due to the interaction of 11-MUA. The amide II band (C-NH), which appeared at 1563 cm⁻¹ in DesfB was shifted to 1533 cm⁻¹ upon iron chelation. This shifting of the FTIR bands to the lower wavenumber corresponds the bond formation. The band at 1167 cm⁻¹ that was exhibited in the DesfB spectra, assigned to one type of CN vibration, disappeared in the spectra of Ferrioxamine B. Multiple small bands in the range of 859–968 cm⁻¹ for DesfB were in Ferrioxamine B at 859–1068 cm⁻¹. Due to the transformation of the DesfB to its iron chelated part, there have the evidences of overlapping bands.

FTIR spectrum of DesfB bound to 11-MUA SAM is shown in Fig. 2. 11-MUA on Au has C=O band formation at 1650 cm⁻¹ which further shifted to 1608 cm⁻¹ and formed a strong band upon DesfB interaction. The appearance of a strong band at 1608 cm⁻¹ instead of multiple bands demonstrates the bonding between NH₂ group of the ferric complex of DesfB and COOH group of the 11-MUA. The C-N stretch and N-H bend vibration at 1726 cm⁻¹ and C-H vibration at 1423 cm⁻¹ appears upon iron chelation. Binding between iron chelated DesfB coated Au surface with both live and dead E. coli bacteria is shown in Fig. 3. The DesfB coated surface only allows live bacterial cells to be attached and it is evident through FTIR band formation. In presence of live E. coli cell, the band at 1608 cm⁻¹ disappeared with appearance of three new bands at 1550 cm⁻¹, 1640 cm⁻¹ and 1673 cm⁻¹ assigned to amide I and II. These bands appear due to the attachment of cell surface protein receptor of live E. coli cell with DesfB coated surface.

The FTIR spectra of DesfB and dead cell treated surface looks similar without any new bond formation or any changes demonstrates that upon dead cell treatment no bond was formed in between them. The band intensity at 1608 cm⁻¹ became reduced upon dead cell interaction. A new shoulder band arises at 1750 cm⁻¹ assigned to C=O which might be from the iron chelated DesfB complex.

**Fluorescence microscopy analysis.**—The microbial cell is considered viable if the membrane is intact and it can be assumed to be dead if the membrane structure is ruptured. In the experiment both the dye SYTO 9 and Propidium Iodide (PI) were equally mixed and treated with bacterial cells to check the membrane structural integrity. Both the dyes have their own staining properties to distinguish viable and non-viable bacterial cells. PI was added to the SYTO 9 mixture having different concentration of live/dead cells. Fig. 4a indicates the

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**Table I. Consolidated ATR-FTIR spectral wave numbers of DesfB and iron chelated DesfB molecule in a tabular format.**

| Frequency (wavenumber cm⁻¹) | DesfB | Iron chelated DesfB | Band assigned |
|---------------------------|-------|--------------------|---------------|
| 3317                      | 3287  | N-H of the secondary amide |
| 3110, 3133                | 3087  | C-N-H overtone      |
| 2960                      | 2950  | asymmetric CH₃ stretching |
| 2960                      | 2950  | C=O                |
| 1563                      | 1533  | C-NH, amide II     |
| 859–968                   | 859–1068 | N-O stretching     |
viable cells showing green color due to the penetration of dye SYTO9. PI penetration induces red color emission of dead cells observed in Fig. 4b. Both green and red color emitting cells corresponds to mixture of live and dead bacteria as well (Fig. 4c). The plain DesfB coated (Fig. 4g) and Human IgG coated (Fig. 4h) glass slides were devoid of any emission, which indicates the sample does not have any background fluorescence from DesfB, Human IgG, and their interaction (as negative control). However, live bacteria are critically dependent upon iron sequestration. Iron is the key ingredient for survival and live bacteria synthesize and release siderophores in the culture media to bind iron with a remarkably high affinity. Iron-bound siderophores are recognized by specific cell surface receptors of bacteria. This is a highly conserved process that occurs only in intact bacteria.

The experiment was designed such a manner that DesfB coated glass slide is used to specifically capture viable bacteria from a mixture of viable and dead E. coli, as demonstrated by fluorescence microscopy. Fig. 4d depicts live E. coli bacterial cells decorated on DesfB coated glass slide. The image shows green cells uniformly distributed on the slide surface. In the case of dead E. coli cells coated on DesfB surface (Fig. 4e), the red coloration was missing indicating the absence of binding with DesfB. An interesting result was found in mixed equal population of live and dead E. coli cells treated on DesfB coated surface (Fig. 4f). From Fig. 4f, it shows a green emission zone which indicates that the DesfB coated surface can differentiate live cell from the dead one and further it concludes that DesfB coated surface can bind only to live cell.

Upon further time lapse, core shell like structure formed where the core and shell is denoted by green and red color respectively. Even in between this structure there is the existence of green emission zone which resembles the presence of a live bacterial cell. It indicates that live cells have the capability to attach with the DesfB coated surface. The red color emitting shell zone indicates the destruction of the outer membrane whereas the green color core demonstrates the intact viable cells.

**Microcantilever study.**—Frequency tracking was performed for the 19th resonant mode of the iron chelated DesfB immobilized microcantilevers (Fig. 5). DesfB coated microcantilever was taken as background i.e. a stable baseline was allowed to be achieved for each DesfB coated microcantilever before the sample was injected. Since the liquid environment is DI water, there was no shift in frequency observed when DI water was injected. However, when E. coli (10⁻¹ dilution) dispersed in DI water was injected shift from the baseline frequency (Δf) is clearly visible due to the E. coli attaching to DesfB coated microcantilever surface. Live and dead E. coli of different

![Figure 4](image_url)

**Figure 4.** Fluorescence Microscopy Images (A) Live E. coli, (B) Dead E. coli, (C) Mixed live and dead E. coli, (D) Desf B + Live E. coli, (E) Desf B + Dead E. coli, (F) Desf B + Mixed Live and Dead E. coli, (G) Plain Desf B and (H) Human IgG on Desf B coated surface.

![Figure 5](image_url)

**Figure 5.** Tracking of 19th resonant mode of microcantilever. Different responses of microcantilever to DI water and E. coli.
Figure 6. Shift in resonance frequency vs time for different injected samples on desfb functionalized SAM immobilized gold coated silicon microcantilever.

Table II. Biomolecular mass loaded for the respective sample injected, calculated from micro cantilever resonance frequency shift.

| Sample Dilution | Live E. coli Δm (ng) | Dead E. coli Δm (ng) |
|-----------------|-----------------------|----------------------|
| 10^-1           | 2.28 ± 0.82           | 0.56 ± 0.25          |
| 10^-4           | 0.69 ± 0.11           | 0.47 ± 0.27          |
| 10^-7           | 0.27 ± 0.05           | 0.15 ± 0.15          |
| Negative control (antibody) | 0.18 ± 0.15 |

original stock), the live cell Δm is 0.27 ± 0.05 ng (386 ± 71 bacterial cells), which is lower than that of dead cell Δm of the previous two dilutions and also, on level with the negative control (0.18 ± 0.15 ng) (257 ± 214 bacterial cells). In earlier section it is already mentioned that in 10^-7 dilution (3.1 × 10^6 CFU/ml bacteria of original stock) there is only 310 bacterial cell present whereas from cantilever study it shows 386 ± 71 bacterial cells. Thus, this system of differentiation between live cell and dead cell is valid up to 10^-4 dilution. In this current study we are less concerned about the number of bacterial cells as the objective of the work is to demonstrate the binding capability followed by sensing activity of Siderophore (DesfB) toward live E. coli cell compare to dead one. The authors did not investigate long-term stability of the platform. However, the platform appears to be stable for at least 3 months (during the experimental testing period) with reliable detection of live bacteria.

Conclusions

In summary, the recent work demonstrated immobilization of Siderophores (DesfB) on solid surfaces such as a cantilever surface to distinguish between live and dead E. coli. The use of self-assembled monolayer based gold thiol chemistry for surface functionalization helped attach DesfB covalently on top of the gold coated surface. FTIR study revealed the interaction between live bacterial cells to the DesfB coated surface. Simultaneously this attachment was further observed by Fluorescence microscopy image analysis. The live cell attachment to the DesfB coated surface has been reported and validated by bacterial cell mass differential. It is seen that live cells attach to the functionalized cantilever whereas dead cells do not have the tendency to do so.

This study can serve as a preliminary demonstration toward experimental data in constructing a biosensing platform to differentiate bacterial versus viral illness in real time for point-of-care diagnostics aiding Internet of Things (IoT) application.

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References

1. W. L. Jodi, N. A. Mutalib, K. Chan, and L. Lee, Frontiers in Microbiology, 5, 1 (2015).
2. M. Collin and R. Schuch, (Eds.), Bacterial Sensing and Signaling, Karger (2009).
3. P. Calo-Mata, M. Carrera, K. Bohme, S. Caamano, J. M. Gallardo, J. Barros, and B. Canas, J. Anal. & Biosanal. Tech., 7, 1 (2016).
4. S. A. Bustin, V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, and G. L. Shipley, Clinical Chemistry, 55, 611 (2009).
5. Z. Kuo, D. Richard, D. Andrea, D. Dominik, and M. Erwin, Toxins, 6, 1325 (2014).
6. J. B. Neilands, The Journal of Biological Chemistry, 270, 26723 (1995).
7. M. Colin and G. D. Lynn, Annual Review of Microbiology, 54, 881 (2000).
8. C. H. Robert and K. Xiaole, Nature Product Reports, 27, 637 (2010).
9. L. W. Mark, S. A. Aaron, P. Lakshman, G. S. Jurgen, and M. Harshini, Advances in Biological Chemistry, 2, 396 (2012).
10. D. D. Doorneweerd, W. A. Henne, R. G. Reifenberger, and P. S. Low, Langmuir, 26, 15424 (2010).
11. E. Hashem, K. M. F., K. Kamaljit, and T. Thomas, Nature Communications, 7, 1 (2016).
12. L. Bogdan, W. Aleksandra, and R. Zenon, PLoS One, 12, 1 (2017).
13. J. W. Ndieyira, M. Watari, A. D. Barrera, D. Zhou, M. Vogti, M. Batchelor, M. A. Cooper, T. Strunz, M. A. Horton, C. Abell, T. Rayment, G. Aeppli, and R. A. Mckendry, Nature Nanotechnology, 3, 691 (2008).
14. W. Jingjing, Z. Yinfang, W. Xing, W. Shuaineng, Y. Jinling, and Y. Fuhua, IEEE Sensors Journal, 16, 4675 (2016).
15. L. Stephane and L. Yongjun, A cantilever biosensor exploiting electrokinetic capture to detect Escherichia coli in real time, Sems and Act B, 238, 292 (2017).
17. T. E. Clarke, V. Braun, G. Winklemann, L. W. Tari, and H. J. Vogel, *Journal of Biological Chemistry*, **277**, 13966 (2002).
18. P. Bhadra, M. S. Shahjahan, E. Bhattacharya, and A. Chadha, *RSC Advances*, **5**, 80480 (2015).
19. A. E. Martell, R. M. Smith, and R. J. Motekaitis, *Critically selected stability constants of metal complexes database*. NIST standard reference database, **46** (1997).
20. P. Borer, S. J. Hug, B. Sulzberger, S. M. Kraemer, and R. Kretzschmar, *Geochimica et Cosmochimica Acta*, **73**, 4661 (2009).
21. H. Siebner-Freibach, S. Yariv, Y. Lapides, Y. Hadar, and Y. Chen, *Journal of Agricultural and Food Chemistry*, **53**, 3434 (2005).
22. P. Bhadra, M. K. Mitra, G. C. Das, R. Dey, and S. Mukherjee, *Materials Science and Engineering: C*, **31**, 929 (2011).