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Selective Imaging of Quorum Sensing Receptors in Bacteria Using Fluorescent Au Nanocluster Probes Surface Functionalized with Signal Molecules

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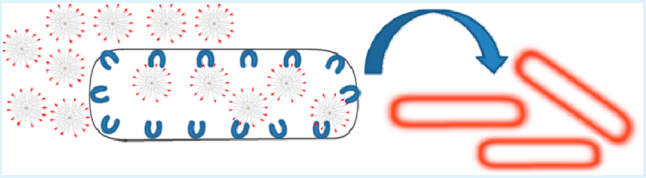
Supporting Information

ABSTRACT: Fluorescent ultrasmall gold clusters decorated with bacterial quorum sensing signal molecules, acyl homoserine lactone, are synthesized. These fluorescent probes are found to have emission in the near-infrared spectral region advantageous for bioimaging. Imaging studies using different strains of bacteria with and without acyl homoserine lactone receptors with the aid of confocal microscopy have shown that the probe interacts preferentially with cells possessing these receptors. This indicates that, with appropriate surface functionalization, the Au clusters can be used for receptor specific detection with enhanced selectivity.



KEYWORDS: *quorum sensing, Au nanoclusters, fluorescence imaging, biosensor*

INTRODUCTION

Quorum sensing (QS) or the phenomenon of bacterial communication has been identified and studied in detail for more than a decade now.[1−3] Such kind of intercellular communication allows bacteria to come together and function as a single entity more or less like a multicellular organism.[4] Quorum sensing is suggested to be the mechanism by which biofilm formation takes place, which enhances the virulence, drug resistance, etc., of various bacterial pathogens. QS is mediated by small easily diffusible signal molecules which regulate target gene expression and is highly dependent on the density of bacterial cells in the medium. Different categories of molecules mediate quorum sensing in different strains of bacteria which can be loosely categorized into those in gram negative and gram positive bacteria.[5] Gram positive bacteria depends on autoinduction by small peptides and in gram negative bacteria, QS is mediated by acylated homoserine lactone (AHL) class of molecules.[6,7] These autoinducers are perceived by their complementary receptors. and this process is highly selective with respect to the signal molecule and receptors. Binding of autoinducers to the specific receptor sites triggers cascade processes regulating a wide range of phenomena often detrimental to the host organisms.[8−10] In case of AHL autoinducers, receptors belong to Lux-R family of transcription regulators and they are usually located intracellularly or may be found attached to the inner leaflet of bacterial cell membrane.[11]

Even though lot of biochemical information has been brought to light in recent studies regarding the mechanistic aspects of quorum sensing, attempts to visualize this phenomenon have been rare. Recently, imaging the phenomenon of quorum sensing in gram negative organisms, specifically *Pseudomonas aeruginosa* (PAO1) has been attempted, employing in vivo approaches whereby a quorum sensing biosensor strain was used as a probe to detect the signal.[12] However, this method is dependent on growth of both the test strain and the biosensor. Such growth associated methods are time-consuming and carries the risk of a full blown infection of the host in the meantime, hence do not seem appealing enough when urgent detection of bacterial pathogens is required. Imaging and identifying bacterial pathogens at low cell numbers, before they reach a quorate state and cause havoc, seems highly desirable. Such an early detection can be envisaged if we target the receptors which are present in the bacterial cells even before the virulent quorate phase. Very recently, CepR quorum sensing receptors in live cells of *Burkholderia cenocepacia* have been tagged using signal molecules which are terminally labeled with fluorescent organic moieties.[13] However, inorganic nanomaterial based fluorescent probes emitting in near-infrared (NIR) spectral region are understood to be superior to organic dyes because of their higher photostability, reduced background fluorescence etc.[14] Imaging quorum sensing by such inorganic nanomaterial based fluorescent probes is as of yet unexplored. In this scenario, a

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simple, photostable and selective fluorescence imaging of quorum sensing based on receptors facilitating early detection of bacteria will be advantageous. On the basis of this, we have developed an inorganic gold nanomaterial fluorescence imaging probe selective to bacteria possessing AHL receptors, *Escherichia coli*. *E. coli* is a gram negative bacterium which perceives AHL signal molecule through LuxR family of receptors SdiA[15] but does not produce the signal molecules. This method seems to have potential for species selective imaging of quorum sensing in vivo with the added advantage of being capable of detecting bacterial cells much before the virulent stage and at lesser populations.

EXPERIMENTAL SECTION

Synthesis of Au nanoclusters is reported by us elsewhere.[16] Typically, 8.5 mL of a stock solution of $HAuCl_4$ (99.9%, Aldrich) in acetonitrile (10 mM) was mixed with 20 mL of acetonitrile. Thiol (0.593 g, 1.6 mmol) in 2 mL of methanol was added to this mixture. The mixture was stirred for 30 min. One hundred microliters of $NaBH_4$ solution (1.58 M) in methanol was added and stirring was continued for 45 min. All the steps were carried out in ice-cold conditions. Thirty milliliters of water was added to gold nanocluster solution and acetonitrile was removed in vacuum. Aqueous solution of gold nanocluster was purified by dialysis using cellulose membrane (12 kD Aldrich). The concentration of this final solution was found to be 2 mM of Au by elemental analysis. Thirty microliters of a stock solution of oleic acid in methanol (0.6 mM) was mixed with 1 mL of the above gold nanocluster solution. Ten microliters of AHL (99%, Cayman Chemicals) in 500 μL of ethanol was added to this mixture and stirred for 4 h. This was considered as the stock solution for incubation with bacterial cells. For intermediate nanoclusters, same volume of either AHL or oleic acid was added to 1 mL Au−thiol solution.

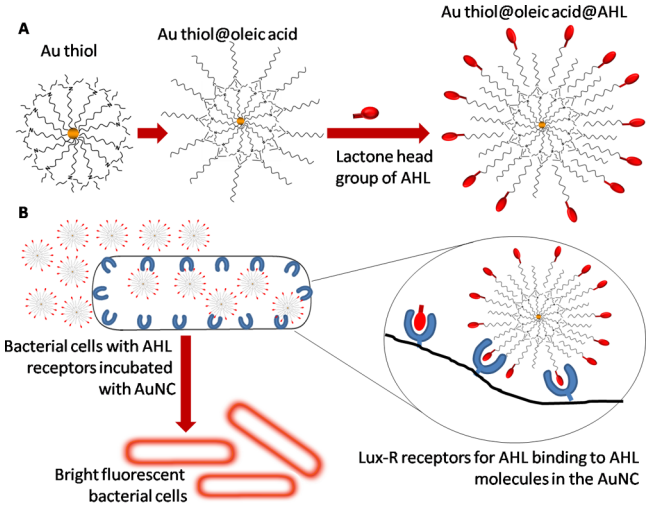
FEI Tecnai TF-30 electron microscope, operating at 300 kV was used for high resolution transmission electron microscopy (HRTEM). Samples for HRTEM were prepared by evaporating a droplet of solution onto a carbon coated copper mesh 200 grid. UV−vis spectra were recorded on a Cary 5000 UV−vis−NIR spectrophotometer. Fluorescence emission spectra were obtained on a Photon Technology International Fluorescence Instrument. Samples were taken in 3.5 mL quartz cells of 10 mm path length for fluorescence measurements. Two detectors were used: (1) R2658 PMT in PTI cooled housing with a single em mono, 1200 L/mm, 400-nm blazed grating; (2) InGaAs diode with chopper and locking and a single em mono, 600 L/mm, 1250-nm blazed grating. The spectra were corrected for the sensitivity of the respective emission channel and normalized. Quantum yield was calculated based on experiment in a small integrating sphere and was done using a double/single (excitation/emission) monochromator format. The lamp used was a xenon arc lamp with a chopper set for 27 Hz. The detector was a 1700 nm InGaAs. The grating used was 600 L/mm, 1.25 μm blaze angle. The bandpass was 12 nm for excitation and 48 nm for emission. FTIR spectra were recorded on a Perkin-Elmer FT-IR spectrum GX instrument. KBr crystals were used as the matrix for preparing samples. Olympus Fluoview Laser Scanning Microscope was used to observe the stained bacterial cells. Confocal microscopy slides were prepared using 70% glycerol as mounting medium and this did not hamper with the fluorescence of the sample. Coverslips containing the sample and mounting medium were sealed at the sides to prevent drying by evaporation and slides were observed within 3−4 h of sample preparation.

RESULTS AND DISCUSSION

Inorganic semiconductor quantum dots like CdSe, HgSe, etc., and Au nanoclusters (NCs) are reported to be advantageous with emissions in NIR region thereby minimizing interference from endogeneous fluorescence of the sample media.[17−19] In case of Au nanomaterials, as the particle size decreases to <2 nm forming ultrasmall clusters, the electronic properties deviate from those of the bulk material and start exhibiting molecule like orbital characteristics which manifest as unique physical properties like fluorescence.[20] NIR fluorescence emissions of these materials render them ideal for bioimaging. The major drawback of Au NCs is their low quantum yield (QY); however, Lin et al. has demonstrated the capability of low QY AuNCs as fluorescence probes for specifically targeting human hepatoma cells.[21] Hence, with appropriate surface functionalizations, a widespread application of AuNCs in the field of sensing and imaging of a variety of biologically important systems can be envisaged. The fluorescent probe under study consists of water dispersible AuNC with size <2 nm initially layered with oleic acid (OA) and subsequently coated with AHLs of chain length C6 and C8. *Chromobacterium violaceum* mutant strain (CV026) based bioassay confirmed that the biological activity of AHL signal molecules is not lost despite being directly coated over a nanoparticle. This is the first report of such retention of activity by AHL molecules. CV026 is a biosensor organism used in QS studies because of its ability to respond to a variety of synthetic AHL molecules. Purple pigment (Violacein) production in CV026 is a QS-mediated phenotype and exogenously supplied AHLs can induce its production. When the Au NC composite was incubated with CV026, violet pigment production was observed indicating that chemistry used in synthesizing this AuNC-AHL conjugate did not in any way destroy the inherent QS biological activity of the AHL molecules. Additionally, no enzymatic or lipolytic activity inherent to the mutant biosensor strain CV026 was responsible for release of AHL molecules from the AuNC-AHL conjugate leading to eventual violacein production. This was confirmed by growing the culture in basal mineral medium with oleic acid as the sole source of carbon at a concentration of 0.1%. No growth was observed after incubating the culture for 48 h at 30 °C under shaking conditions. This shows that the violacein is produced upon perception of the conjugate as a whole and not AHL alone that has been removed from the conjugate by enzymatic cleavage.

The strategy employed here involves surface functionalizing fluorescent Au NCs with AHL in such a way that capability of the signal molecules to bind to specific receptors is not compromised. Initially, fluorescent Au nanoclusters are synthesized using an alkyl thiol ligand, *N,N,N*-tripropyl(11-mercaptoundecyl)ammonium chloride with a cationic ammonium headgroup to facilitate dispersibility in water, most conducive solvent for biological systems.[16] The AHL signal molecules consist of lactone headgroup and a long chain alkyl group of varying lengths (structures of AHL molecules under study, C6-AHL and C8-AHL, with six and eight C alkyl chains are given in Supporting Information). It is understood that ring carbonyl and 1-carbonyl groups of cognate AHL molecules bind to the N-terminal receptor sites of Lux R proteins through hydrogen bonding.[22] Hence it is imperative to leave the lactone and amide moieties intact and free to interact with the receptors. However, if pristine Au-thiolate cluster is added directly, there is a possibility of interaction between cationic head groups of NC and the lactone moiety of AHL. Hence, further surface functionalization of the NCs is necessary to avoid any cooperative interaction between lactone and cationic headgroup of AuNCs. A composite was designed such that outer surface of the NC would form a hydrophobic region which would then interact with the alkyl chains of AHL molecules (Scheme 1A). Accordingly, NCs were initially treated with oleic acid so that electrostatic attraction between

Scheme 1. Interaction of the Fluorescent Probe with Bacterial Cells: (A) Structure of the Probe with AHL Signal Molecules Deployed on the Surface with Lactone and Amide Moieties Intact and (B) Specific Binding of AHL Head Groups to Receptor Sites in Lux-R Regulators within Bacteria[a]



[a]Binding sites shown schematically; actual location is not known.

cationic head groups and COOH groups was facilitated. Such a binding would allow the hydrophobic alkyl chains of oleic acid to decorate the outer surface of NCs rendering them ideal for interaction with the long chain alkyl groups of AHL. In this way, the lactone would be free to attach to the receptors in bacterial cells (Scheme 1B).

The Au NCs were found to be fluorescent in NIR region (Supporting Information) with an emission maximum at 840 nm (Quantum yield -3%). The average number of thiol molecules attached to the Au clusters was roughly estimated based on number of surface Au atoms and a methanol solution of oleic acid of equimolar concentration was added. Subsequently, lactones with varying chain lengths, namely, C6 and C8 were mixed with the above Au-thiol@oleic acid composite. Transmission electron microscopy revealed ultra-small clusters of size <2 nm, functionalization not affecting the particle size (Supporting Information).

The fluorescence emission also was found to be similar with emission maximum at 860 nm and quantum yield of 1.6% (Supporting Information). Interactions between various functionalizing molecules were ascertained by solid state FTIR spectroscopy. Electrostatic interaction between carboxylic acid group and ammonium headgroup based on carboxylic C═O functionality was followed in comparison to pristine oleic acid. It is known that this acid C═O stretch which is observed in 1710 cm^{-1} in pristine sample disappears in bound states with appearance of bands near 1640 and 1540 cm^{-1} corresponding to symmetric and asymmetric −COO vibrations.[23] We could also observe a strong band at 1636 cm^{-1} corresponding to symmetric and a very weak band at 1523 cm^{-1} corresponding to asymmetric −COO stretch (Full assignment in Supporting Information). This indicates a possible configuration of the cluster with COO^- group interacting with ammonium group thereby deploying the alkyl functionality on the outside. IR spectrum of the composite after addition of C6-AHL in comparison to methanolic solution of AHL is given in Figure 1.

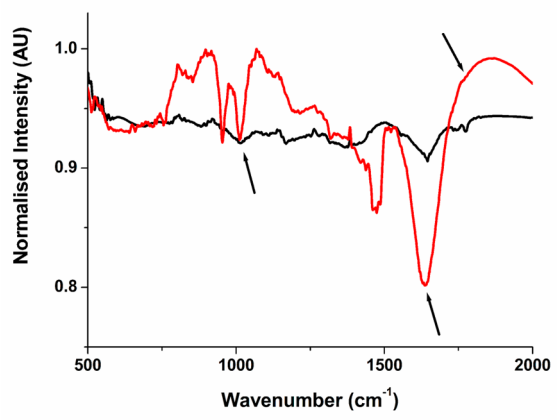


Figure 1. FTIR spectra of ethanolic solution of C6-AHL (black) and solid Au@OA@AHL (red); arrows indicate amide and lactone group bands which are intact in the composite.

It can be seen that amide group bands are more prominent than lactone bands in both.[24,25] In case of pure AHL, amide group bands consist of a strong C═O stretch band at 1646 cm^{-1} and weak N−H bend at 1520 cm^{-1}. Possible lactone C═O stretch could be seen at 1772 cm^{-1} and C−O stretch at 1167 and 1014 cm^{-1}corresponding to O−CO and $O-CH_2$ vibrations. After addition of AHL, the lactone group bands were intact at 1772 and 1011 cm^{-1} indicating that the bioactive region is free to interact with the receptor sites. O−C═O vibrations cannot be unambiguously assigned due to overlap with Au-OA bands. With these evidence we could tentatively conclude that the composite has the structure Au-thiol@oleicacid@AHL (Au@OA@AHL) with the lactone moiety on the outside of the cluster. Moreover, the surface charge was found to be negative with a zeta potential value of −38 mV indicating that the conjugate is highly stable (Supporting Information).

We selected representative bacteria from two distinct groups, one gram negative (*Escherichia coli*) and one gram positive (*Staphylococcus aureus*) for further fluorescence imaging. Quorum sensing receptors in *E. coli* are of the Lux-R family but it is interesting to note that this strain does not produce AHL molecules and *S. aureus*, being gram positive does not contain the receptors. Initially, bacterial cells were suspended in saline and incubated with the Au@OA@AHL conjugates for 2 h with gentle shaking. After incubation cells were pelleted down, washed with saline and resuspended in fresh saline so that any unbound fluorescent clusters would not interfere with the imaging. All experiments were carried out using low cell numbers of the order of 10^6 cells/mL well below the quorate number of any bacterium. These cells in glycerol medium were observed under confocal microscope at preset excitation and emission wavelengths of 547 and 567 nm respectively.

In case of *E. coli*, Au clusters decorated with both C6 (Supporting Information) and C8-AHL (Figure 2) showed bright fluorescent emission and cell contours could be easily identified. Supernatant solution without the cells also showed emission, however, in irregular agglomerated shapes pointing to unbound clusters (Supporting Information). Since AHLs are specifically produced by gram negative bacteria, rationally the Au NCs decorated with AHL molecules should only interact with cells of *Escherichia coli* and not with *Staphylococcus aureus*, the gram positive bacterium with a different receptor system. To establish this hypothesis, in a separate experiment, cells of

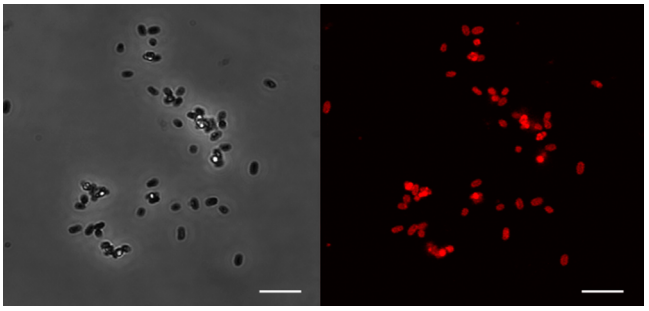


Figure 2. Confocal microscopy images of *E. coli* incubated with Au@OA@C8-AHL: (left) phase contrast image and (right) fluorescence image of the same region. Scale bar is 5 μm.

stained *S. aureus* were observed under same conditions as *E. coli*. As expected, *S. aureus* did not show fluorescence indicating that it failed to interact with Au@OA@AHL as displayed in Figure 3. This shows the inherent specificity of this conjugate

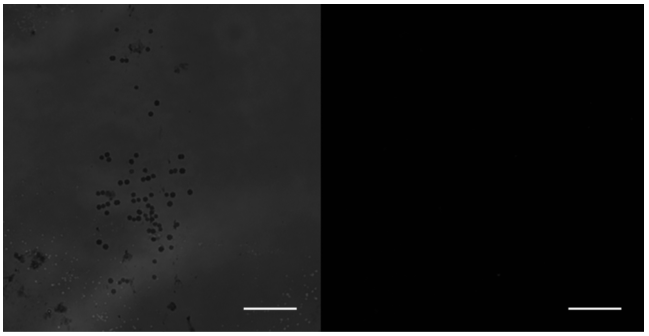


Figure 3. Confocal microscopy images of *S. aureus* incubated with Au@OA@C8-AHL: (left) phase contrast image and (right) fluorescence image of the same region. Scale bar is 5 μm.

to interact with only those cells which possess receptors for AHLs. This indicates that the probe conjugates are capable of accessing the receptor sites within the bacterial cells. Moreover, the negative charge of the probe rules out any electrostatic interaction with the bacterial walls.

To further test our hypothesis of the structure of the composite and mode of binding, *E. coli* cells were also stained with two intermediate NCs, namely, Au-thiol@AHL and Au-thiol@oleic acid. These NCs were synthesized in such a way that concentrations of Au-thiol as well as AHL and oleic acid were same as that of the Au@OA@AHL conjugate. Former sample represents the situation where outer surface of Au NCs is not hydrophobic so that lactone moiety may interact with cationic ammonium headgroup of the thiolate layer. In such a case, we envisage nonavailability of the binding sites of the signal molecule and hence no detection of the bacterial cells. The latter indicates a similar case whereby a hydrophobic Au NC without the signal molecule is used. It is possible that the fluorescent Au NCs can indiscriminately access the receptors even without the signal molecules, in which case Au-thiol@oleic acid should bind to bacterial cells. Interestingly, no fluorescence could be seen (Figure 4) in either of these cases giving credence to the proposed structure model. This clearly indicates that the structure of the fluorescent probe is Au@OA@AHL with the lactone moiety decorating the surface which can interact with the receptors on bacterial cells.

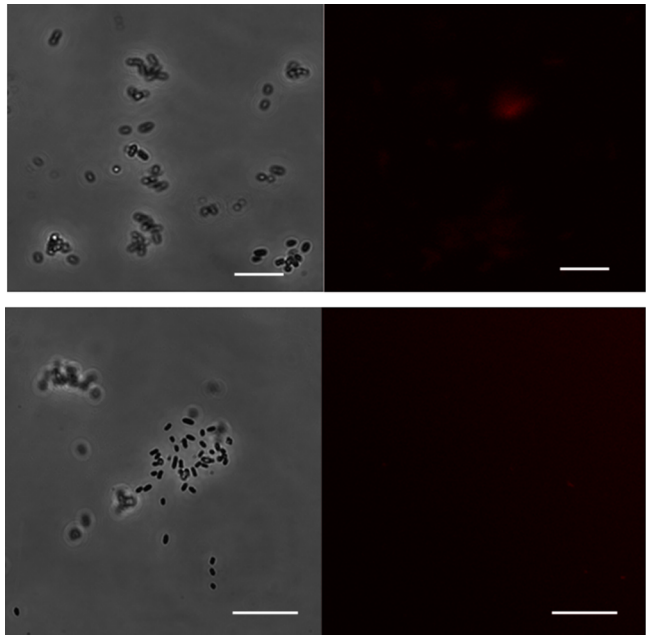


Figure 4. Confocal images of *E. coli* incubated with Au-thiol@C8-AHL (top) and Au-thiol@oleic acid (bottom): (left) phase contrast image and (right) fluorescence image of the same region. Scale bars measure 10 μm.

It was also found that in a mixed population of *E. coli* and *S. aureus*, gram negative bacterial strain *E. coli* could be exclusively observed under fluorescence imaging (Figure 5). This could be

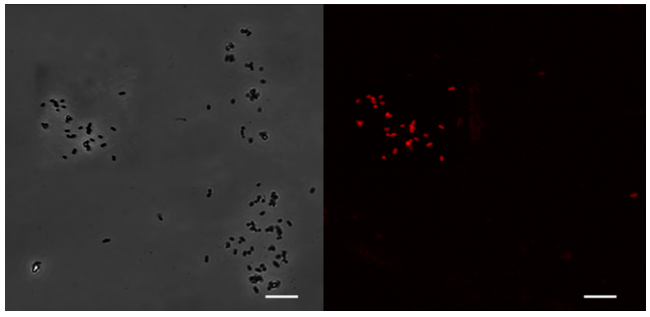


Figure 5. Confocal images of mixed population of *E. coli* and *S. aureus* incubated with Au@OA@C8-AHL: (left) phase contrast image and (right) fluorescence image of the same region. Scale bar is 10 μm.

identified due to the shape difference between the two strains of bacteria from the phase contrast image, emphasizing the enhanced specificity of the probe. An interesting point worth highlighting is that *E. coli* cells do not produce AHLs but are able to detect and respond to these QS signal molecules by eventual gene regulation. Specific detection of *E. coli* by our probe system further highlights the fact it is the possession of AHL receptor that is imperative and not the growth stage of cell or population density for this system to function as a potential biosensor.

To ascertain the interaction mechanism, we selected another gram negative bacteria *Chromobacterium violaceum* (wild type) which is a natural producer of AHL molecules. The logic here is that if the receptor sites are already interacting with AHL in the system after the threshold concentration of signal molecules, this will prevent the interaction of the fluorescent probe to these sites. In such a case, bacterial cells incubated with the

conjugate should not show any fluorescence activity and indeed this was observed (Figure 6). Also, another test was carried out

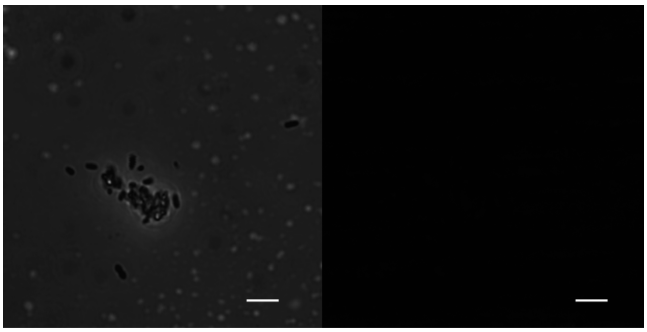


Figure 6. Confocal images of *Chromobacterium violaceum* (wild type), which produces AHL incubated with Au@OA@AHL: (left) phase contrast image and (right) fluorescence image of the same region. Scale bar is 2 μm.

in which *E. coli* cells were first incubated with excess of free AHL so that the receptor sites are saturated and then this system was incubated with Au@OA@AHL conjugates. The confocal image does not show any indication of interaction by the probe to the bacterial cells (Supporting Information). This indicates that the receptor sites were not available for binding to Au@OA@AHL conjugate due to their saturation with excess free AHL initially added. These two observations prove that the AHL component of the conjugate interacts with the receptor sites specifically and also that these AHL molecules are strongly bound to the fluorescent Au-thiol nanocluster. From these studies, we can also rule out any breakage of the conjugate structure. However, the latter observation may point to a limitation of this material in detecting bacterial strains which produce AHLs at a fully quorate state because of preferential binding of free AHLs compared to those conjugated to the AuNC system.

CONCLUSION

In summary, a novel inorganic fluorescent probe capable of selectively binding to receptor sites involved in quorum sensing is developed. The material is based on Au nanoclusters decorated with quorum sensing signal molecules for gram negative bacterial strains, acyl homoserine lactones. The structure was designed such that the bioactivity of the signal molecule, vis-á-vis their lactone and amide groups, is intact after interacting with the fluorescent Au nanoclusters. This probe targets the binding sites for QS molecules within bacterial cells and not the concentration of signal molecules produced which is the current practice. This property makes these systems independent of cell density and can be used before the bacteria attain a virulent quorate state. This is the first time such inorganic fluorescent probes are used for bacterial detection before quorate state and the excellent specificity for binding sites renders this system indeed ideal for targeted biosensors. The simple synthetic method employed would make it easy to design other such receptor specific fluorescent probes which can be employed in complex biological samples after appropriate sample processing. This technique has the potential to be used in detection of pathogenic as well as environmental bacteria. Further exploration into widening the detection species and understanding of specific binding mechanisms will pave way to more efficient early detection techniques.

ASSOCIATED CONTENT

Supporting Information

Chemical structures of AHL molecules, extra confocal images, IR spectra, TEM images, and PL spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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

Notes

The authors declare no competing financial interest.

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