Rapid Magnetic Nanobiosensor for the detection of Serratia marcescens

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Abstract The development of rapid, sensitive, accurate and reliable bacterial detection methods are of keen interest to ensure food safety and hospital security. Therefore, the development of a fast, specific, low-cost and trusted methods is in high demand. Magnetic nanoparticles with their unique material properties have been utilized as a tool for pathogen detection. Here, we present a novel iron oxide nanoparticles labeled with specific targeting antibodies to improve specificity and extend the use of nanoparticles as nanosensors. The results indicated that antibody labeled iron oxide platform that binds specifically to \textit{Serrattia marcescens} in a straightforward method is very specific and sensitive. The system is capable of rapid and specific detection of various clinically relevant bacterial species, with sensitivity down to single bacteria. The generic platform could be used to identify pathogens for a variety of applications rapidly.

Keywords (Bacterial detection, Iron Oxide Nanoparticles, Antibody Detection, Sensor)

1. Introduction
In recent years, bacterial detection from various sources is of great interest in the medical, environmental and food manufacturing. Among these bacteria that present a significant threat to human health are; \textit{Campylobacter}, \textit{Salmonella}, \textit{Listeria monocytogenes}, \textit{Escherichia coli} (\textit{E. coli}) O157: H7, \textit{Staphylococcus aureus}, and \textit{Bacillus cereus} (1-5). Therefore, it is of great importance to developing reliable and sensitive methods for bacterial detection. Many approaches have been reported in the literature for the bacterial detection, including the culture and colony counting, polymerase chain reaction (PCR), and immunology-based methods (6, 7). The conventional detection mainly relies on colony counting method. This is a practical way for the detection and identification of pathogens from different resources, including bacterial culturing and isolation of the pathogen. Moreover, bacterial detection requires further identification by the confirmation using biochemical and serological experiments, which takes up to 5–7 days to get a result (5, 8). Although the methods above can obtain a reliable result, it is labor intensive and time-consuming, which cannot satisfy the request for on-the-spot bacterial detection. The PCR and enzyme-linked immunosorbent assay (ELISA) is a lot less time-consuming than the traditional culture and colony counting method (8). However, and to overcome some of the difficulties mentioned with the conventional methods the search for new alternative more reliable and sensitive methods lead to the development of the field of nanotechnology.
Nanomaterials offer an exceptional opportunity to revolutionary impact on biology and medicine because of the size-dependent physical and chemical properties of the nanoparticles.
Nanomaterials have been exploited extensively to provide targeted drug delivery, diagnostics, tissue regeneration, sensing tools, antimicrobial agents, and molecular imaging. Various platforms like, liposomes, dendrimers, quantum dots (QD), fullerenes, virus-like particles, and carbon nanotubes are being developed to improve human health (9-12). Magnetic nanoparticles (NPs) hold great potential and currently is under investigation in magnetic hyperthermia treatment (13), drug delivery (14) and cell sorting (15). Paramagnetic iron oxide NPs used as contrast agent in Magnetic Resonance Imaging (MRI) and photothermal therapy against cancer (16). Fe₃O₄ magnetic NPs used for liver imaging, a contrast agent for MRI, immunoassay, biosensing and drug delivery (17, 18). Silica-coated iron oxide PEG-coated NPs with contrast element Gadolinium (Gd) used to access a specific area of the brain to detect a tumor(15, 19).

Iron oxide coated NPs (IONPs) exhibit excellent biocompatibility, and they are considered a very stable colloid. IONPs labeled with antibodies have been used to detect breast cancer cells in vitro (20). In addition, IONPs conjugated with luteinizing hormone-releasing as breast cancer cells used to detect breast cancer in vivo (21). NPs are promising agents for antibacterial applications because of the selective toxicity against bacteria like silver NPs. Furthermore, some antibacterial nanoparticles can be degraded by lysosomal fusion and thus appear non-toxic to mammalian cells (22). In addition, a variety of moieties has been examined as targeting agents, including carbohydrates, peptides, receptor ligands and proteins such as transferrin and lectins (23, 24).

Bacterial infections are the fifth cause of mortality in the US with considerable economic impact on healthcare sector (25, 26). Traditionally, the presence of a bacterium in any sample is detected microscopically, usually after growth in culture. Confirmation is based on growth patterns in various media and biochemical tests. These methods, although highly specific, have some drawbacks. First, they usually take more than 24 hours and lack the desired sensitivity due to some bacteria needed, while not all microorganisms can grow in culture. In addition, other methods involving polymerase chain reaction (PCR) and fluorescent immunoassays have been developed for bacterial detection. However, despite their enhanced sensitivity and specificity, these methods are expensive, time-consuming, and need to be performed by trained and experienced personnel.

One of the methods that have been developed which holds a promising potential is that the direct immobilization of antibodies on the surface of an electrode for the detection of bacteria called impedimetric immune-sensors. This relies on the immobilization of antibodies on the electrode surface and then probing the attachment of the bacterial cells by measuring the change in electrical properties of the electrode (8). Antibodies have long been the most popular recognition elements because of their sensitivity and selectivity (27, 28).

Nanotechnology and nanoparticles (NPs) offer an attractive alternative for the identification of molecular targets in vitro and in vivo. Biosensors based on nanomaterials have been used to amplify detection signal and achieve lower detection limit due to their high surface area (29, 30). The use of NPs requires smaller samples volumes and less preparation time, without compromising the detection sensitivity (31, 32). Over the past decade, superparamagnetic iron oxide nanosensors have been designed to quantify biomolecular targets in cell lysates and tissue extracts, demonstrating high sensitivity (33, 34). The underlying detection method utilizes various molecular targets such as nucleic acids (DNA and mRNA), antibodies, peptides and other chemical moieties.

The interaction between the NPs and the microorganisms are often toxic and has been exploited for various antimicrobial applications. However, investigating the interactions and the selectivity of specific bacterial detection is of great importance in the field of bionanotechnology. The existing...
detection methods for bacteria that are available relied on ELISA, Western Blots, surface plasmon resonance (SPR) biosensors, antibody microarrays Ref. The mentioned methods require high doses for the sample detection although some are sensitive the need for faster and more reliable method still in need. The method that is reported here is prompt, fast, reliable and relatively inexpensive. The development of nanotechnology offers many technological advances for the detection of bacterial. This paper acknowledges the need for the development of fast and sensitive bacterial detecting biosensor. In addition, we investigate the use of IONPs for the detection of the bacterium in solution. Modifying NPs with the target antibody as shown in Figure 1, in this case, will lead to the decoration of the bacterium surface with the modified IONPs, the high number of NPs that are expected to bind to the surface of the bacterium would lead to higher sensitive's, particularly al low target bacteria concentrations.

Figure 1 Schematic representation of the modification methods approach. The IONPs were decorated with antibodies that are unique to the bacterial model.

As a proof of this hypothesis, we used Serratia marcescens as our model organism. We selected this bacterium as a proof-of-principle model since it belongs to the family, Enterobacteriaceae, which is a large family of Gram-negative bacteria that includes, several pathogens, including Salmonella, Escherichia coli, Yersinia pestis, Klebsiella, Shigella, Proteus, Enterobacter, Serratia, and Citrobacter. This family belongs to the order Enterobacteriales of the class Gamma proteobacteria in the phylum Proteobacteria. It was originally considered to be a harmless, non-pathogenic, organism and it was frequently used as a biological marker because of its easily distinct red colonies (Merlino, 1924). Serratia marcescens has been recognized as an important opportunistic pathogen combining a propensity for healthcare-associated infection and antimicrobial resistance. It is a widely distributed saprophytic bacterium and has been found in food, particularly in starchy variants which provide, an excellent growth environment (35). Currently, 14 species of Serratia marcescens are recognised within the genus, eight of which are associated with human infection (36). Intensive care units are frequently involved in the epidemics of infection with Serratia marcescens. It accounts for less than 1-2% of the hospital-acquired infections which are frequently limited to the respiratory tract, the urinary tract, surgical wounds and soft tissues (37, 38). The nanosensor used in this study was prepared by the conjugation of anti-Serratia antibody (Details) to superpara magnetic IONPs. The antibody labeled nanoparticles
acted as very stable months, after storage at 4°C. Apart from sensitive and fast detection, the
detection method is independent of the samples optical properties.

2. Materials and Methods
All general reagents, iron salts (FeCl$_2$, 4H$_2$O and FeCl$_3$, 6H$_2$O), dialysis membrane 14 kDa, NaOH,
epichlorohydrin, purchased from Sigma-Aldrich, Dextran (10 kDa) were received from
Amersham Biosciences/Pharmacosmos and Sigma-Aldrich, succinic anhydride, N-
hydroxysuccinimide, sodium hydroxide (NaOH), epichlorohydrin, NH$_4$OH, standard
concentrates 10.00 g Fe (Sigma CAS No 10421-48-4), Anti-Serratia marcescent antibody B/N4N
was obtained from Abcam, Distilled water, DMSO, EDC: 1-ethyl-3-(3-dimethyl aminopropyl)
carbodiimide hydrochloride, NHS: N- hydroxysulfosuccinimide sodium salt.

2.1. Bacterial Culture
*Serratia marcescens* (ATCC 27117) pure culture provided by the laboratory of microbiology and
biotechnology, Department of Biological Sciences, Yarmouk University-Jordan; were grown on
nutrient agar media plate. The bacterial strain was inoculated into the nutrient broth, and on
nutrient agar media, the resulting pure colonies were used to prepare bacterial suspensions. A loop
full of the bacterial strain was used to inoculate nutrient broth and was incubated for 24hrs at 30
°C. The bacterial suspension turbidity was adjusted to 0.5 McFarland standards. One millilitre of
this bacterial suspension was transferred to a new tube of broth and incubated at 30 °C for six
hours before each experiment. The serial dilutions of bacterial culture were prepared using
sterilized broth water before each experiment.

2.2. Synthesis of IONPs
IONPs were synthesis following adapted protocol adapted from reference (39). Briefly, a mixture
of FeCl$_2$ (3 mM) and FeCl$_3$ (6 mM) was dissolved in 100 mL Milli-Q or DD-H$_2$O water and
prepared freshly before use. Dextran 10 kDa (2.5 g) of technical grade dextran (Sigma-Aldrich, Cat.
Nr. D9260, lot Nr. BCBK8656V) dissolved in 5 mL Milli-Q water and was placed on a rotary
spinner until a clear solution is formed. Iron chloride solutions with a molar ratio of 2:1 as
described earlier was then mixed with a freshly prepared aqueous solution of dextran and stirred
vigorously for 30 minutes under constant N$_2$ gas circulation. The pH of the solution was adjusted
slowly up to pH 12 using 10 mM NaOH solution by drop wise addition. As an indication of the
IONPs formation, the solution colour changed accordingly from yellow to dark brown to black.
The solution was incubated at 80°C (Thermoblock or water-bath) for 2 hours. Samples were then
transferred over a magnetic stirrer and mixed using the magnetic stirrer for one hour at ambient
temperature. The solution was purified from excess dextran by dialysis overnight (17 hours)
against DD-H$_2$O using dialysis membrane 14 kDa (Sigma-Aldrich) while exchanging water every
2 hours (3 times) and then left overnight at ambient temperature. The resultant purified NPs were
concentrated using spin columns, and the iron content was determined spectrophotometrically
(DAR800-Diagnostic automation, INC, USA).

2.3. Synthesis of amine terminated dextran coated NPs
The particles were prepared following adaptation from (40) 20 mL of the previously prepared
dextran covered NPs were placed into a 250 mL round flask equipped with a magnetic stirrer at
the ambient temperature. 36.7 mL of NaOH (5M) was added as a drop wise (roughly at a rate of 1
drop per second). After that, 13.5 mL of epichlorohydrin was added to drop by drop rate. The
mixture was stirred for 7 hours at ambient temperature followed by the addition of 20 mL of NH$_4$OH (25%) was added at drop wise fashion, and then placed on a magnetic stirrer for 14 hours at ambient temperature. The mixture was dialyzed against water through a dialysis membrane 14 kDa Spectra-Por Float-A-Lyzer G2 bags (Sigma-Aldrich). The mixture was stirred for overnight and then dialyzed against DD-H$_2$O.

2.4. Synthesis of carboxyl terminated dextran coated NPs

20 mL of dextran-coated iron oxide nanoparticles were placed in 100 mL flasks equipped with stirrer bar for 5 minutes at an ambient temperature to homogenize the solution. A freshly prepared aqueous solution of 30 mg of succinic anhydrate was added in two stages to maintain pH (~ 5) of the reaction. The mixture was stirred for 1 hour at an ambient temperature, after that, another aqueous solution containing 30 mg of the succinic anhydrate was added. The mixture was stirred overnight and stored at 4°C until needed.

2.5. Preparation and Purification of bacterial suspension for TEM images

Bacteria were grown overnight in 10 mL of nutrient broth in a shaker incubator at 30°C. Followed by the addition of 1.5 mL of the bacterial broth addition an Eppendorf tube and centrifuged for 5 minutes at 10,000 g. The supernatant was discarded, and the pellet was washed twice with sterile distilled H$_2$O and resuspended using vortex. This bacterial suspension was viewed and examined as well as for the control under TEM.

2.6. Examination and Imaging Bacterial Suspension under TEM

5–10 µl of 0.1 mg ml$^{-1}$ suspended particles were allowed to settle briefly (1 minute) onto pyroxylin and carbon–coated copper grids (300 mesh, Agar Scientific), and then blotted dry with filter paper. Grids were viewed at 60 kV in an EM10CR TEM (41). TEM images were performed by CCD microscopic camera (Optikam B9 Digital Camera).

2.7. Sectioning and Imaging under TEM

Bacterial cells were incubated with the antibody labeled NPs. Cells were fixed in a solution of 2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer (pH= 7.3) for two h at 4 °C. Fixed cells were washed three times with 0.1 M sodium cacodylate buffer each wash for 5 minutes. Post-fixation staining was done using 1 % stock solution of osmium tetroxide for 1 hour. This was followed by washing with 0.1 M sodium cacodylate for 1.5 hours for three times each wash is for 5 minutes at 4 °C. Cells were dehydrated in acetonitrile (30, 50, 70, 90 and 100%) and infiltrated in Spurr’s low viscosity (3:1, 1:1 and 1:3) resins. Then, cells were infiltrated in pure Spurr’s resin (Low Viscosity Embedding Media Spurr's Kit, Electron Microscopy Sciences) for 12 hours, embedded in Beam capsules containing pure resin blocks, and finally hardened at 70 °C for 17 hours (overnight). Ultrathin sections (70-90 nm) were cut by Ultra microtome (Reichert-Jung) and the sections were loaded on copper grids (300 mesh, Agar Scientific) and stained using 5% (w/v) aqueous uranyl acetate. Samples were then analyzed under a 60 Kv ZeissEM10CRTEM microscope, TEM images were performed by CCD microscopic camera (Optikam B9 Digital Camera).

2.8. Dynamic light scattering (DLS)

DLS was measured on a DynaPro Titan, Wyatt Technology Corporation (laser wavelength 830 nm, scattering angle 20 degrees) with Dynamics software Version 6.9.2.11. Particles at a
concentration of 0.5–1 mg m⁻¹ cm⁻¹ in 10 mM sodium phosphate buffer pH 7.0 were filtered through 0.1 – 0.4-micron filters (Millipore) before analysis. Four independent measurements were performed; each single measurement presents an average of ten measurements. Data were recorded at 20 °C.

2.9. Surface charge measurement
Zeta potential was measured on a Malvern Instruments Zetasizer–Nano ZS where 1 ml of 0.5 mg ml⁻¹ particles were suspended in 10 mM sodium phosphate buffer pH 7.0. Zeta cells were equilibrated at 21°C before recording three measurements each of 12 runs. The data were fitted using the Smoluchowski approximation assuming Henry’s function f(Ka) of 1.5.

2.10. Preparation and purification of bacterial and IONPs.
The prepared bacterial culture as described earlier was then spun at 10,000 g for 5 minutes. The supernatant was discarded, and mixed the pellet with 300 µL of IONPs and suspended using vortex. This bacterial-IONPs suspension was viewed and examined under TEM.

2.11. Determination of Iron content
Spectroscopic measurements are most easily performed with liquid samples, and the protocol is adapted from (42). To convert the insoluble Fe₂O₄ in the unknown (NPs) to a soluble species, an acid digestion was performed. They have used a solution of Fe³⁺. The spectroscopic methods used here are based on a molecular spectroscopy procedure that is based on forming complexes between the iron with an organic ligand and measuring the absorbance of the resulting complex. In this experiment, measurements were recorded by generating a calibration curve of prepared (known) standards of Iron atomic spectroscopy standard concentrates 10.00 g Fe (Sigma CAS No 10421-48-4) and the formation of a linear calibration model between the absorbance (X-axis) and the concentration (Y-axis) (mg/mL). The calibration curve is designed with the unknown concentration falls somewhere in the middle of its range. Ten µl of aqueous 3% freshly prepared an H₂O₂ solution and ten µl of each standard solution was added to 1 mL of 5 M HCl. The NPs particles to be tested were prepared by taking the ratio of 1/3 of IONPs solution to 2/3 Milli-Q water, in triplicate two µl of the nanoparticles from the dilution and incubate with two µl of aqueous 3% freshly prepared an H₂O₂ solution and 200 µl of 5 M HCl. The NPs particles were incubated at 50 °C for 1 hour in a Thermoblocks with the same amount of 1 mL of 5 M HCl. The standard curve and the sample absorbance were measured on 96 well plates in triplicate at 410 nm. The measured absorbance was subtracted from the background, and the standard curve was plotted on excel sheets. The iron content was calculated from the slope of the standard curve generated.

2.12. Antibody labeling NPs
Carboxylic acid groups were activated by the carbodiimide method using 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC-Thermofisher Scientific) and NHS (N-hydroxysulfosuccinimide-Sigma) or sulfo–NHS. Carboxyl terminated IONPs suspended in either 10 mM or 0.1 M sodium phosphate buffer pH 7.4 were incubated with a freshly prepared Milli–Q water solution of EDC (1 molar equivalent) for 5 minutes followed by the addition of a freshly prepared dimethylsulfoxide solution of NHS dissolved in DMSO (4 molar equivalents). The reaction was allowed to proceed for 2 hours at room temperature with gentle stirring. The resulted in O–succinimide esterified IONPs were purified by 10-14 kDa MWCO dialysis membrane. The esterified particles were then incubated with the anti-Serratia marcescens monoclonal antibody
(100 µL at 2 mg/mL stock concentration in which 20 µL of antibody were added directly to solution mixed contain EDC, NHS and carboxy NPs) and the reaction was left to proceed overnight while gently shaking at 4°C. Antibody-labeled IONPs were then purified on PD–10 desalting columns (Amersham) were used according to the manufacturer’s instruction and were pre-equilibrated with ten mM sodium phosphate buffer. Eluted samples were collected and further analysed.

Figure 2 schematic representation of the antibody conjugation strategy on the exterior surface of the IONPs.

2.13. QuantiPro Bicinchoninic acid (QPBCA)
Bicinchoninic acid (BCA) protein assay was chosen as the second protein quantification method. The technique relies on the reduction of Cu$^{2+}$ to Cu$^{+}$ by proteins leading to the formation of the purple-blue copper protein complex in alkaline conditions that can be monitored spectrophotometrically at $\lambda=562$ nm. Protein concentrations in the presence or absence of MNP were measured with a BCA Protein Assay Kit as described by the manufacturer instructions (Sigma-Aldrich). Briefly, 200 µl of BCA reagent was added to each sample. Samples were incubated at ambient temperature overnight while gently shaking at 100 rpm. The absorbance of the reaction products was measured at $\lambda=562$ nm using a microplate reader (Tecan).

2.14. Bradford protein assay
Protein quantification was determined following Bradford assay method relying on the binding of Coomassie brilliant blue G-250 to the conjugated protein the IONPs resulted in the shift in the absorption maximum of the compound dye from 465 to 595 nm. The increase in the absorption at 595 was determined photometrically as described by Bradford (43). In essence, a 1 ml aliquots of freshly prepared Bradford reagent was added to 100 ml of IONPs and antibody labelled IONPs; samples were carefully mixed. A 200 µl aliquots of the solution above was transferred to a 96-well-plate. The reaction left to proceed for 45 minutes at ambient temperature, the sample absorbance was determined spectrophotometrically at $\lambda=595$ nm (Tecan).

3. Results and discussion:
3.1. The characterisation of IONPs
The particles were characterised for every step of the synthesising procedure and before modifying the NPs. The synthesized particles were characterized by TEM and DLS instrumentations. The dextran coated DLS determined NPS with an average of ~60 nm from five independent syntheses, and each set of measurements includes ten replicates. In addition, TEM observations revealed that the morphology and the size of the iron core are ~20 nm surrounded by the dextran coating up-to 60 nm in diameter as shown in Figure 3. The polydispersity index of the synthesized IONPs is 0.002 % which indicates ‘monodisperse’ distribution of the particles. It has been reported
that nanoparticles with < 0.20 % polydispersity index are considered “highly monodisperse (44, 45). Also, the correlation plot as shown in Figure 2, confirms the absence of aggregation in the sample and indicating the particles are a stable colloid.
Figure 3 Size distribution intensity graph of IONPs nanoparticle as revealed by DLS with different surface functionalities with its corresponding correlation fit (A) dextran coated IONPs (B) Carboxyl terminated IONPs (C) antibody labeled IONPs
Zeta potential values are another useful tool for the stability of colloidal particles and confirming the successful surface modification. The absolute values reflect the net electrical charge on the particle surface. The suggested theoretical limit of stability is $|25|$ mV. Particles are considered to exist as stable colloids of their zeta potential is higher than 25 mV or lower than –25 mV (46, 47).

The zeta potential analyses were conducted for all the modification steps to confirm successful modification of the NPs surface charge. The Z-potential of the dextran-coated nanoparticles were be found on average -0.165 (see Figure 4-A), while the carboxyl terminated nanoparticles (negatively charged) were found to be ranged from -10.7 to -26 as shown in Figure 4-B. In addition, Z-potential were utilized to confirm the surface modification of the IONPs with the antibody; the surface charge values indicated a value of $-53.8 \pm 8.0$ mV see Figure 4-C. In addition, the zeta potential values suggested that the particles are very stable colloid and no propensity to aggregate, which is in agreement what the visual inspection of the samples as no visible aggregation have been seen on any of the prepared samples. Particles were stable for 2-3 months with no visible particles aggregation.
Figure 4 Zeta potential measurements of the functionalized IONPs (A) Dextran coated IONPs (B) carboxyl terminated IONPs (C) antibody labeled IONPs

TEM analyses of the synthesized IONPs revelled a solid core of the size 10-12 nm with a monodisperse size distribution and no visible aggregation in all samples synthesised as shown in as shown in Figure 5.
Figure 5 TEM image of unstained IONPs showing monodisperse particles distribution and solid metallic core of 10-12 nm in diameter.

TEM samples for this work were prepared by a typical process of fixation, dehydration, and embedding in a resin matrix has been reported in the literature (48, 49). TEM was utilized as a tool to examine non-sliced and sliced bacterial cells before the incubation with the IONPs and then examine the binding profile of antibody labeled IONPs elimination the electrostatic interactions as the only mean for the specific bindings as shown in Figure 6.
Figure 6 unstained and fixed section of 80 nm in thickness of Serratia marcescens as viewed by TEM. The yellow arrow indicates lysed or empty bacterial cell. White arrow indicates cell wall-free cytoplasmic content.

The TEM images gave valuable information on how and where the NPs were about the bacterial cells. To visualize the surface interactions between the NPs and the bacterial model, sectioned TEM images were acquired to confirm the binding of the antibody labeled NPs on the bacterial surface. TEM images of Serratia in the presence of 10 µg of iron as determined by the iron content. The lower magnification images show the overall morphology of cells and NPs, while Figure 7 at higher magnification reveal more specific interactions between cells and NPs. Overall, for all the nanoparticles interaction work, no internalization of IONPs was observed into bacterial cells.

The TEM images show that upon incubating IONPs with the bacteria, Serratia cells remain intact with very minimal to the non-noticeable extent of IONPs on the bacterial cell. The lack of nonspecific association (binding) between the antibodies labeled IONPs was confirmed. The TEM revealed non-specific binding of the main carboxyl terminated IONPs in comparison to the amine terminated ones as seen in Figure 9. During TEM sample preparation IONPs-bacteria was pelleted after 10 minutes’ incubation period. Followed by multiple steps of buffer rinse, the majority of antibody labeled IONPs that were not cell-bound were washed off in the supernatant, leaving the Serratia pellet free of unbound particles before embedding and sectioning. Figure 7, demonstrate that where cell surface binding occurred, a small cluster of antibody-IONPs was partially attached to the cell surface at various points, without compromising the integrity of the cell. The majority of the visible IONPs formed uniformly packed with a thin layer of the IONPs around the cells. An interesting antibody labeled IONPs interaction pattern was observed. IONPs labeled particles remain well separated, neatly lining the cell surfaces while keeping a small gap between the IONPs and the cell wall. Although the reason for this phenomena is not clear, one hypothesis that has been...
published in the literature is that the NPs surface chemistry that leads to aggregation also plays a vital role here. The uniform gap between the attached IONPs and cell walls could be either the result of LPS that does not give significant TEM contrast or a double-layer (50). Overall, no internalization of IONPs was observed into bacterial cells from any of the TEM images. It is clear that IONPs were attached to the thick peptidoglycan layer of the cell wall, far from the buried lipid membrane layer. Furthermore, to investigate whether the observation from the EM images is actual IONPs. The EM grids were further analyzed by Energy-dispersive X-ray spectroscopy (EDX-INCA Energy 200Premium attached to a JEOL JSM 5900LV scanning electron microscope-SEM), the data confirmed that the layer that is surrounding the EM images are composed of iron as shown in the inset of Figure 8.

Figure 7 TEM graph of antibody labelled IONPs conjugated to Serratia marcescens after rigours purification. The particles are attached to the carbohydrate layer on the bacterial surface.
Figure 8 TEM of the antibody labeled IONPs conjugated to Serratia spp with the elemental analysis in the inset. The EDXs confirmed that the layer surrounding the bacteria consists of the iron signal in the selected area.

Whole cell imaging of Serratia with an antibody labeled IONPs showing the attachment of the IONPs on the outer layer of the bacteria. EDX analysis confirmed the presence of iron which is in agreement of the successful binding of the IONPs to the bacterial surface. TEM studies of the antibody labeled IONPs provided a snapshot of the localization interactions. The non-specific binding of the amine terminated-IONPs, carboxy terminated-IONPs, and the antibody labeled IONPs were investigated. The amine terminated IONPs some affinity towards the cells. The cationic coating on the IONPs surface induced limited non-specific binding. The non-specific binding could be attributed to the presence of negative charge on the surface of the IONPs based on the comparisons with control samples as seen in Figure 9 and 10. This could be hypothesized through the electrostatic interaction between the cationic IONPs and the negative cell surface, leading to cell wall attachment to higher degrees than the anionic IONPs. Also, the TEM sample preparation procedure involving repeated pelleting steps may have artificially enhanced the attachment observed in these images. Furthermore, the ultra-thin (70-90 nm) sections of the same antibody labelled bacterial samples that were used for the whole particles studies revelled that the IONPs were present attached to the cells or outside. None of the examined sections did show any visible IONPs within the bacterial cells confirming that the binding is to the exterior of the cells.
Furthermore, the antibody quantification was determined spectrophotometrically in the presence and the absence of the antibodies at $\lambda = 562$ nm, which was quantified via BCA and determined to be $1.5 \pm 0.2$ mg/ml iron. Bradford method for the estimation of the protein content showed no net absorbance change at $\lambda=595$ nm. All measurements on both methods were in agreement and within the experimental errors of the protein conjugated to the IONPs as shown in Figure 11. To demonstrate the magnetic properties of the IONPs-labelled with the antibody the labelled particles were put close to Neodymium Magnet which resulted in pulling the labelled bacteria to the side of the tube as shown in figure 12.
Figure 11 Absorbance spectra of antibody conjugated to IONPs after the incubation with the Bicinchoninic acid (BCA) reagent overnight. Spectra were recorded at $\lambda = 562$ nm. All assays were performed in triplicates; absorbance has been registered with the standard deviation as the error in the measurements.

![Absorbance spectra](image1.png)

Figure 12 image of the IONPs labelled bacteria (A) that move with an applied external magnetic field (B).

![Image of labeled bacteria](image2.png)

It has been reported in the literature that, a TEM study examining non-sliced bacteria interactions with six nm-diameter cationic AuNPs reveled AuNP clusters on the Bacillus surface could be dispersed upon the removal of surface proteins by trypsin (51). These results may contribute
toward our understanding of the molecular-level identification of cellular components responsible for NP interactions with bacterial cells.

The growth curve of Serratia under normal conditions depicted the lag, log, stationary and death phase (data not shown). It is believed that the bacterial growth was not affected by the addition of the IONPs has been confirmed through the incubation of the IONPs with different surface moieties on agar growth plates.

4. Conclusions
Bacteria are vital contributors and single cell model organism for evaluating the impact of nanomaterials in the environment. Herein, we report a very simple, easy and reliable method for the selective detection of bacteria of interest from any sample. The specificity of antibodies was combined with magnetic properties of the nanoparticles enhanced the detection capabilities of bacteria detection. TEM studies reveled that no NPs were internalized by the bacterial strain. IONPs functionalized with the antibody reveled the highest binding toward the bacteria. These results demonstrate the importance of a thorough understanding of the IONPs targeting.

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