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BIOSENSORS

Fluorescent Gold Nanoparticle-Based Bioconjugate for the Detection of *Salmonella*

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ABSTRACT

An easily prepared fluorescent bioconjugate colloidal solution based on gold nanoparticles is reported for the detection of *Salmonella*. This bioconjugate was prepared by conjugation of staphylococcal protein A on the surface of gold nanoparticles, followed by biofunctionalization over this layer with fluorescein isothiocyanate-labeled antibodies. The surface plasmon resonance band observed in the ultraviolet–visible spectrum of the gold nanoparticles showed a shift to low energy after covering the surface with protein A and a broadening of the band after biofunctionalization with the antibody. Surface-enhanced infrared spectroscopy showed the absorption bands of the conjugate and bioconjugate due to their proximity to the nanoparticles. In particular, vibrations at 1649, 1588, and 1400 cm^{-1} were from C=O, N–H, and C–NH_2 groups from the staphylococcal protein A covering the gold nanoparticles. The final bioconjugate colloidal solution was also used as probe to detecting *Salmonella* by confocal microscopy of solutions containing 1E3 and 1E5 CFU/ml. The total time required to detect these bacteria was less than 1 h. Fluorescent emission of the bacillus suggests that this methodology may have potential to develop a universal method for detecting different bacteria.

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Introduction

The presence of microorganisms in food is a natural and unavoidable occurrence. Cooking generally destroys most harmful bacteria, but undercooked foods, processed ready-to-eat foods, and minimally processed foods may contain harmful bacteria that are serious health threats. Meat, dairy, and poultry products are important reservoirs for many of the food-borne pathogens, including *Salmonella, Campylobacter, Listeria, and Escherichia coli O157:H7* (Swaminathan and Feng 1994). *Salmonella* is a common cause of food-borne illness and occurs in raw poultry, eggs, beef, fruit, and vegetables. *Salmonella* is a genus of rod-shaped, Gram-negative, nonspore-forming, predominantly motile *enterobacteriaceae* with diameters from 0.8 to 1.5 μm, lengths from 2 to 5 μm, and peritrichous flagella. Every year, *Salmonella* is estimated to cause about 1.2 million illnesses in the United States, with about 23,000 hospitalizations and 450 deaths. Most persons infected with *Salmonella* develop diarrhea, fever, and

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abdominal cramps 12–72 h after infection (Clark and Barret 1987). Conventional culture methods remain the most reliable and useful techniques for food-borne pathogen detection. However, a major drawback is that these methods are labor-intensive and take 2–3 days for results and up to 7–10 days for confirmation. The elimination of food-borne pathogenic microorganisms is based on plausible policies for sanitary inspection, but this requires development of faster and more effective methods for detecting and identifying hazardous bacteria in those areas where biological hazard constitutes a security problem.

Rapid, selective, and sensitive detection technologies for pathogenic bacteria are critical in clinical diagnosis, disease control, environmental monitoring, and food safety (Wang, Ravindranath, and Irudayaraj 2010). Biosensors offer several advantages over existing techniques that include limited hands-on time, high-throughput screening, improved detectability, real-time analysis, and label-free detection methods and devices (Skottrup, Nicolaisen, and Justesen 2008). The inclusion of nanotechnology into the field of biosensors holds great promise for addressing the analytical needs of medical diagnostic systems (Jain 2007). In this context, gold nanoparticles have raised great expectations with respect to generating enhanced signal-to-noise ratios, reducing response times, and for use in multiplexed systems.

The use of gold nanoparticles for biosensors is a relatively new area of research. Nevertheless, the literature studies already show numerous examples incorporating gold nanoparticles into biodevices (Balasubramanian and Burghard 2006). Nanobiosensors have been reported for the specific detection of nucleic acids (Litos et al. 2009), proteins (Hu et al. 2009), enzymes (Hong et al. 2009), and infectious agents (Liu et al. 2007). Gold nanoparticles are ideal for biosensing assays because of their unique characteristics, namely high solubility in water, and easily tailored synthesis for suitable morphology, size dispersion, and surface functionalities (Baptista et al. 2008). Recently, fluorescence-based approaches have been explored in conjugation to gold nanoparticles towards the establishment of immunoassays (Peng et al. 2007). Gold nanoparticles may cause fluorescence enhancement or fluorescence quenching of molecules in their vicinity, depending on the distance between the nanoparticle and the fluorophore (Dulkeith et al. 2005). It is also known that gold nanoparticles are able to quench fluorescence with larger efficiencies than organic quenchers, and are more stable than the latter (Dubertret, Calame, and Libchaber 2001).

Recent progresses of the gold nanoparticles-labeled antibody or antigen for immunoassay involve either direct binding of antigen-gold nanoparticle bioconjugates to an antibody-modified surface or the exposure of an antibody-derived surface to free antigen and then to a secondary antibody-gold nanoparticle conjugate. The conjugation of antibodies to nanoparticles may generate a product that combines the properties of both (Haiss et al. 2007). They may combine the small size of nanoparticles and their special thermal, imaging, drug carrier, or magnetic characteristics with the abilities of antibodies, such as specific and selective recognition. The hybrid product will show versatility and specificity (Arruebo, Valladares, and Fernández 2009).

Recent reports on the *Salmonella* detection using gold nanoparticles include a gold nanoparticles-based colorimetric assay for rapid detection of *Salmonella* (Prasad, Shankaracharya, and Vidyarthi 2011). They used single-stranded deoxyribonucleic acid probes and nonfunctionalized gold nanoparticles to provide a colorimetric assay for the detection of polymerase chain reaction amplified deoxyribonucleic acid with a total time of less than 8 h. A lateral flow immunoassay was reported for *Salmonella* detection using gold nanoparticles conjugated with a deoxyribonucleic acid probe, which is complementary

to the 16S ribosomal ribonucleic acid and deoxyribonucleic acid of *Salmonella* (Liu et al. 2013). An immunosensor was described for *Salmonella enterica* subsp. *enterica serovar Typhimurium* LT2 detection using a magneto-immunoassay and gold nanoparticles as label for electrochemical detection (Afonso et al. 2013). In a previous paper, we have reported stable gold nanoparticles conjugated with staphylococcal protein A through the estimation of the ultraviolet–visible intensity ratio (aggregate/individual particles) (Ríos-Corripio et al. 2013). This conjugated nanoparticle provides a chemically adequate surface for biofunctionalization with specific proteins and antibodies.

In this article, we report a simple and practical method to obtain a fluorescent bioconjugate to identify *Salmonella* species. This bioconjugate was obtained by using gold nanoparticles which were first conjugated with staphylococcal protein A to give them stability, and then the obtained nanostructures were biofunctionalized with an anti-*Salmonella* antibody. The final bioconjugate colloidal solution was used as a probe to detect the *Salmonella* in contaminated solutions.

Materials and methods

Materials

Tetrachloroauric acid trihydrate 99.5% ($HAuCl_4 \cdot 3H_2O$) was purchased from Sigma-Aldrich, sodium citrate dehydrate ($Na_3C_6O_7$-$2H_2O$) from Baker, and protein A from Sigma-Aldrich. This was stored in pH 7.4, 0.01 M phosphate-buffered saline. Sodium chloride NaCl was purchased from Sigma-Aldrich. A 4.5 mg/ml rabbit polyclonal to anti-*Salmonella* fluorescein isothiocyanate-labeled antibodies were purchased from Abcam (Cambridge, UK). The strain of *Salmonella* was obtained from the Biotechnology Laboratory Technology Monterrey Campus, Puebla. This reference strain has the following identification: *Salmonella enterica* subsp. *enterica serovar Typhimurium* ATCC 14028.

Bacteria Preparation

Salmonella was cultured on agar plate of tryptone soy medium and placed in an incubator at 37°C for 24 h. A single colony was transferred into ten culture tubes containing 5 ml each of tryptone soy broth, under shaking, at 37°C for 24 h. The tubes were centrifuged at 10,000 rpm for 10 min to obtain a pellet. The tryptone soy broth was removed from the tube and the cells were washed thrice with sterile phosphate-buffered saline to remove residual medium and resuspended in 3 ml phosphate-buffered saline for binding experiments. Serial dilutions of bacteria were prepared for the detection step. To validate the data, a standard method for the counting of pathogens was also used: the culture and colony counting method called nephelometry, the concentration of bacteria in the initial nutrient broth was obtained and estimated to be 3×10^9 colony-forming units (CFU)/ml. Serial dilutions were performed in sterile saline solution to 3×10^8–3×10^1 CFU/ml.

Instrumentation

A transmission electron microscope (Philips model Tecnai 10) at an operating voltage of 80 kV was used to analyze the shape and size of the gold nanoparticles. An ultraviolet–visible spectrometer (Evolution 606 Thermo Scientific) was used to measure the surface plasmon

resonance of gold nanoparticles, the conjugate (gold nanoparticles-protein A), and the bioconjugate (gold nanoparticles-protein A-antibody) from 190 to 900 nm.

A Fourier transform infrared spectrometer Bruker Vertex 70 in the attenuated total reflectance (ATR) mode was used to measure the infrared absorption of protein A, antibody, gold nanoparticles, conjugate, and bioconjugate. Conjugate and bioconjugate colloidal samples were centrifuged at 3500 rpm for 40 min; the supernatant was removed and 2 µl of the concentrated sample was placed on the surface of the ATR crystal. The infrared radiation was propagated along the crystal to obtain the corresponding spectrum, which was averaged from several data acquisitions. The infrared spectra were collected from 400 to 4000 cm^{-1}.

A confocal microscope was coupled with an argon/krypton laser emitting at 488 nm to excite the fluorescein isothiocyanate fluorescence was used to acquire the images of the recognition of bacteria by the bioconjugate.

Preparation of gold nanoparticles

Citrate-reduction was used to produce gold nanoparticles (Hermanson 2008) based on the reduction of tetrachloroauric acid by sodium citrate in the presence of heat. The gold nanoparticles are synthesized because the citrate acts as a reducing agent and a capping agent. This method involved the preparation of 1 ml of $HAuCl_4$ at 4% in deionized water. An amount of 0.5 ml of $HAuCl_4$ was added to this solution with 200 ml of deionized water and brought to boiling under constant stirring. Once the sample reaches 97–100°C, 3 ml of 1% sodium citrate was added. As sodium citrate was added, the solution darkened and turned bluish gray or purple. After 30 min, the reaction was complete and the final color of solution was a deep wine red indicating that the colloidal solution of gold nanoparticles was obtained. After the solutions were cooled, the gold nanoparticles were centrifuged at 3500 rpm for 40 min, the supernatant was removed and the nanoparticles were resuspended in 6 ml of deionized water. The obtained suspension was stored in refrigeration at 4°C until use.

Preparation of the conjugate: gold nanoparticles covered by protein A

An amount of 6 µg/ml protein A was used to cover the surface of the gold nanoparticles. In a previous report (Ríos-Corripio et al. 2013), this concentration was the minimum necessary to cover the surface of the gold nanoparticles (for an average particle size of 20 nm), which guarantees stability. The colloidal solution, which contained the conjugate, was centrifuged at 3500 rpm for 40 min and the supernatant was discarded. The pellet was suspended in water and stored at 4°C.

Preparation of the fluorescent bioconjugate: conjugate biofunctionalized with a fluorescein isothiocyanate labeled antibody

An amount of 10 µg/ml of anti-*Salmonella* antibody labeled with fluorescein isothiocyanate was used to functionalize the surface of the conjugated and the colloidal solution was incubated at 37°C for 1 h. The resulting solution was centrifuged at 3500 rpm for 40 min and the supernatant was discarded. The final solution was resuspended in water and stored

at 4°C for infrared spectroscopy analysis and for the recognition of *Salmonella* bacteria with fluorescence microscopy.

Fluorescence detection of Salmonella with bioconjugated nanoparticles

The colloidal solution of bioconjugate nanoparticles was mixed (1:1) with other solutions containing *Salmonella* bacteria (3×10^9 CFU/ml). The mixed bioconjugate-bacteria were incubated at 37°C for 1 h to promote the labeling of the cell membrane of *Salmonella* with the fluorescent bioconjugate. An amount of 5 µl of the obtained suspension was placed on slides covered with poly-L-lysine for characterization by fluorescence microscopy.

Results and discussion

Ultraviolet–visible absorption spectroscopy

The ultraviolet–visible absorption spectra of gold nanoparticles includes a unique and strong band at 520 nm from surface plasmon resonance by the collective electron oscillations of gold nanoparticles (Shi et al. 2012). The intensity and spectral position of the surface plasmon resonance band of colloidal gold are related to the size and shape of the individual structures. Gold nanoparticles form a colloidal suspension, in which there is a balance between the negative charge repulsion and the attractive forces, which may cause coagulation. The addition of electrolytes to the solution may mask the negative charge of each particle and the colloid will begin to collapse as the gold particles adsorb to one another, forming large aggregates and ultimately falling out of suspension. If macromolecules such as proteins are present in the colloidal suspension, thus in place of aggregation and collapse of the suspension, labeling occurs.

In this study, the surface of the gold nanoparticles was covered with protein A to form a conjugate that provides stability to the colloidal system and introduces biocompatible functionalities into these gold nanoparticles for further biological interactions. In a previous paper by Ríos-Corripio et al. (2013), we reported a concentration of 6 µg/ml of protein A as necessary to cover the surface of gold nanoparticles and to obtain a stabilized conjugate system. A new surface plasmon resonance band related with the conjugate particle suffers a spectral shift to low energies (4–6 nm) with respect to the surface plasmon resonance band of individual gold nanoparticles, as shown in Figure 1. This effect is a direct evidence of the binding of protein A with the gold nanoparticles to form the conjugate.

The shift of the wavelength after conjugation of gold nanoparticles with protein A is due to the change in the dielectric constant of the adsorption layer in addition of the increased size of gold particles by the adsorbed protein layer (Nghiem et al. 2010). The ultraviolet–visible spectrum of the protein A (not shown here) in aqueous solution exhibited an absorbance maximum at 276 nm caused by aromatic residues of tyrosine (Sjoholm 1975) and did not interfere with the plasmonic absorption band of gold nanoparticles and conjugate located in the visible range of the spectrum.

Once the gold nanoparticles were covered with protein A, the resulting conjugate particles were functionalized with a polyclonal fluorescein isothiocyanate-labeled anti-*Salmonella* antibody to provide specificity and fluorescence properties. Figure 1 also shows the surface plasmon resonance absorption band from the bioconjugate structure. This band has the same absorption wavelength as the conjugate; however, the signal is much broader

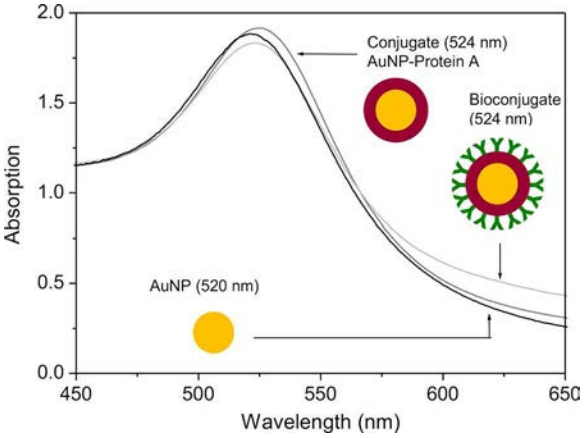

Figure 1. Ultraviolet–visible spectra of gold nanoparticles (AuNP), conjugates (AuNP-protein A) and bioconjugates (conjugate-antibody).

after the functionalization with the anti-*salmonella* fluorescein isothiocyanate-labeled antibody. The layer of protein A that covers the surface of the gold nanoparticles promotes an adequate union of the antibody with the fragment crystallizable because the amino terminal group is exposed by the conjugate (Graille et al. 2000). This is the reason why there is no aggregation after attaching the antibody to the conjugate, allowing the intensity of the surface plasmon resonance absorption be preserved. This feature may be useful to improve the efficiency in biodetection applications.

Transmission electron microscopy

The gold nanoparticles used in this study are shown in Figure 2. They were prepared by the citrate-reduction method and had an average size near 11 nm according to transmission electron microscopy. Figure 3 shows an image of the conjugate composed of gold nanoparticles covered by a layer of protein A. This layer of protein A is responsible of the stability and also

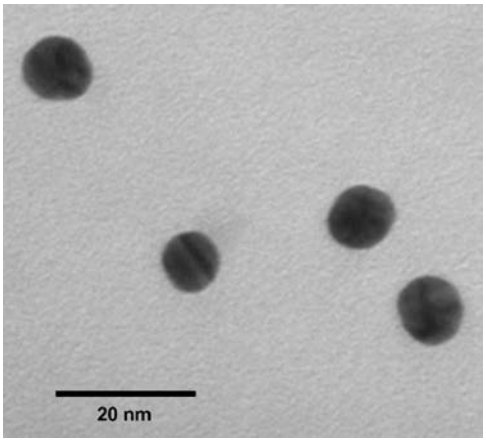

Figure 2. Transmission electron micrograph of gold nanoparticles prepared by citrate-reduction.

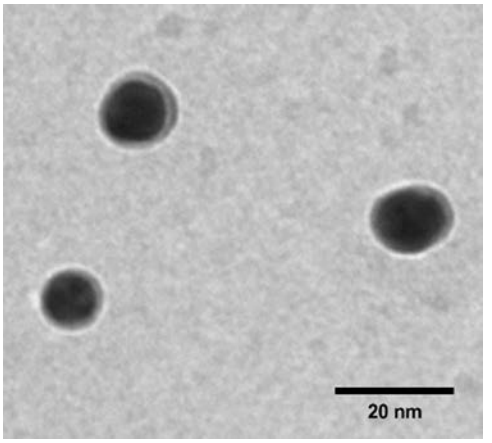

Figure 3. Transmission electron micrograph of the conjugate that includes gold nanoparticles covered with a layer of protein A.

of the adequate attachment of the antibody in the fragment crystallizable region during the functionalization process.

Infrared spectroscopy

The gold nanoparticles, conjugate, and bioconjugate nanostructures were analyzed by infrared spectroscopy and the spectra are shown in Figure 4. In addition, the spectra of protein A and also of the anti-*salmonella* fluorescein isothiocyanate-labeled antibody are included.

The infrared spectra of staphylococcal protein A show an amide I band at 1649 cm^{-1}, which is mainly associated with the C=O stretching (70–85%) and is directly related to the backbone conformation. The amide II band at 1537 cm^{-1} results from N–H bending

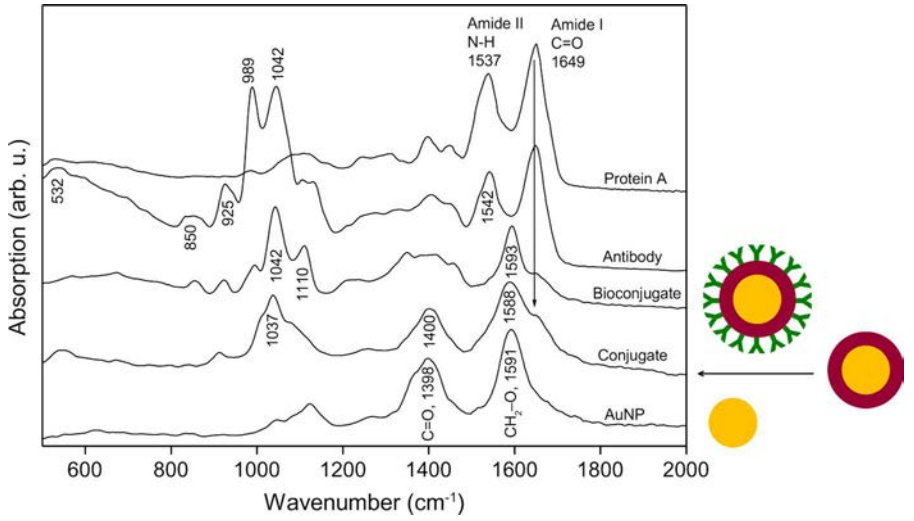

Figure 4. Infrared spectra of gold nanoparticles (AuNP), conjugates (AuNP-protein A), protein A, antibodies, and bioconjugates (conjugate-antibody).

(40–60%) and C–N stretching (18–40%). This band is conformationally sensitive. Amide I and amide II bands are major features of protein infrared spectra. The secondary conformation of protein A is rich in α -helix structure (54%) and also contains 10–20% of the β-structure and a large part of coil structure. Other peaks include bending CH_2 modes (1448 cm^{-1}); stretching C–NH_2 of side chain primary amines near 1400 cm^{-1}, and various weaker bands related to C–N, C–O, and C–C–O vibrations of the protein backbone and amino acid residues (under 1350 cm^{-1}).

The infrared spectrum of the anti-*Salmonella* fluorescein isothiocyanate-labeled antibody shows amide I and amide II bands at 1649 and 1542 cm^{-1} respectively. They have a similar origin as for proteins (Ockman 1978). Other bands at 1110, 1042, 989, 925, and 850 cm^{-1} are associated to the C–O and C–C groups of the glycerol molecule present in the antibody solution. Finally, the band at 532 cm^{-1} corresponds to the backbone S–S link (Sibai et al. 1996).

The infrared spectrum of gold nanoparticles shows two intense bands at 1591 and 1398 cm^{-1} from the CH_2–O deformation and C=O stretching modes from the citrate group as a result of the synthesis (Nghiem et al. 2010). Carboxylated gold nanoparticles are good adsorbents for biomolecules such as proteins; thus molecules of protein A may be linked to the surface of each nanoparticle to form the conjugate.

The infrared spectrum of the conjugate shows drastic differences with respect to the corresponding spectrum of protein A because of the adsorption on the gold nanoparticles (Kamnev et al. 2002). This adsorption was established through the electrostatic interaction between the surface-terminated anionic groups (–COO^-) on the nanoparticles and the positively charged amino groups (–NH+) of the lysine residues of the protein A (Thobhani et al. 2010). In addition, the surface-enhanced infrared absorption effect is also responsible for the observation of the amide I band at 1649 cm^{-1}, because of its proximity to the nanoparticle. The low concentration of protein A used (6 μg/ml) that covers the surface of the gold nanoparticles in the colloidal solution would not be sufficient to detect this protein by using only infrared spectroscopy without the gold nanoparticles. The intense surface-enhanced infrared absorption signal at 1588 cm^{-1} from the N–H vibration is caused by electrostatic interaction between the amino terminal group (–NH+) of protein A with the carboxyl group (–COO^-) located on the surface of the gold nanoparticles. This may indicate that N–H moieties are directly involved in the interaction between protein A and the gold nanoparticles. C–NH_2 vibrations near 1400 cm^{-1} are also enhanced, suggesting the involvement of side chain amino groups, and of C–C/C–O vibrations at 1150–1000 cm^{-1} (Kamnev et al. 2002). According to surface-enhanced infrared absorption theory (Osawa 2001), only those molecular vibrations which appear perpendicular to the metal surface are enhanced, accounting for the selectivity of the enhancement.

The infrared spectrum of the bioconjugate includes several contributions surface-enhanced infrared absorption, such as the C=O stretching of the amide I group from the antibody and protein A at 1649 cm^{-1}. Other signals included a band at 1593 cm^{-1}, which is from N–H contributions of antibodies and protein A. As in the conjugate, vibrations near 1400 cm^{-1} were caused by enhanced C–NH_2 vibrations. Five bands at 1110, 1042, 989, 925, and 850 cm^{-1} were associated with infrared absorption of C–O and C–C stretching of polysaccharides of the antibody used to functionalize the conjugate.

Confocal microscopy

The labeled conjugate obtained in this work in colloidal presentation was mixed 1:1 directly with a solution of water contaminated with a mixture of *Salmonella* and *Staphylococcus aureus* bacteria (10^3 and 10^5 CFU/ml, respectively). After 1 h of incubation at 37°C, a drop of 5 µl of the bacteria mixture was placed on a slide, dried, and analyzed by confocal microscopy. Once the drop dried, the *Salmonella* bacilli and *S. aureus* coccus were observed near the edge of the halo of drop, as shown in Figure 5a and b.

Figure 5c shows the selective recognition of the *Salmonella* bacilli by the bioconjugate prepared in this work. Green emission caused by the fluorescein isothiocyanate chromophore in the bioconjugate is observable in this image and also in the dark field image of Figure 5d. These images show the selectivity of the fluorescent bioconjugate that is able to detect *Salmonella* bacillus. A possible interference of this fluorescent bioconjugate with microorganisms depends on the cross reactivity of the antibody. The selectivity of the bioconjugate colloidal solution for *Salmonella* was high in the presence of *S. aureus*. However,

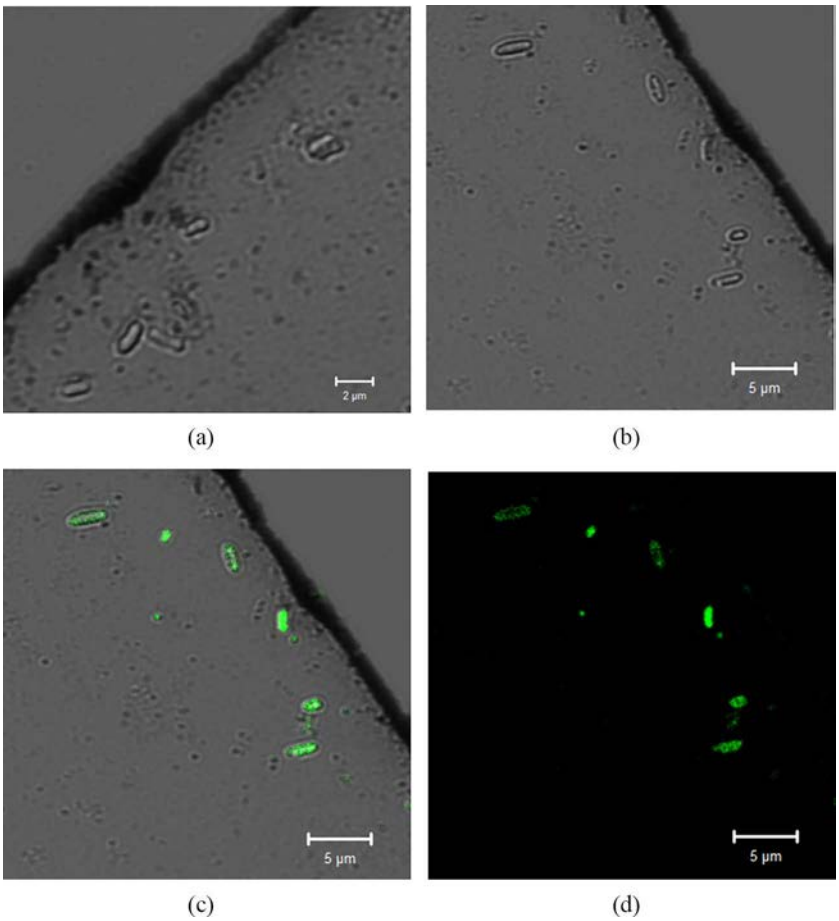

Figure 5. Confocal fluorescence micrographs of *Salmonella* recognized by the bioconjugate in aqueous solution containing *Salmonella* and *S. aureus* bacteria (1E3 and 1E5 CFU/ml, respectively) in (a) and (b) clear-field, (c) superposition of fluorescence and clear-field, and (d) dark-field.

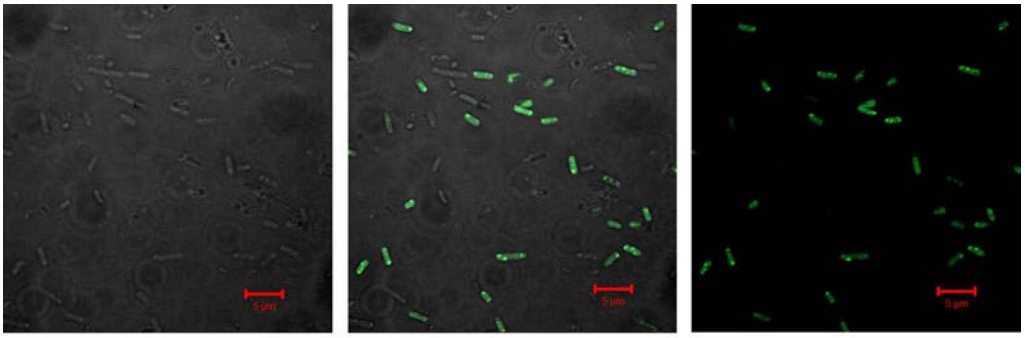

Figure 6. Confocal fluorescence micrograph of *Salmonella* bacteria (1E5 CFU/ml) recognized by the bioconjugate developed in this work. The sample was immobilized on slides covered with poly-L-lysine. Conditions: (a) clear-field, (b) superposition of fluorescence and clear-field, and (c) dark-field.

the selectivity needs to be further investigated to eliminate the interferences from this bacteria and other *Entrobacteria* species, and also to minimize nonspecific binding by using different capture antibody and blocking procedures. This methodology may provide an easy and a reliable way to detect *Salmonella* species in real samples.

A 5 µl of mixed bioconjugate-bacteria at 10^5 CFU/ml were immobilized on slides covered with poly-L-lysine. Fluorescent bacillus is observed in the confocal microscopy images of Figure 6. The use of poly-L-lysine slides constitutes an adequate form to immobilize the bacilli and to obtain fluorescent emission images because the living bacteria cannot move. Figure 6 also shows that some bacilli were less fluorescent than the others, probably by prolonged exposition of the illuminated area to the laser excitation.

Figure 7 shows a fluorescence image of a centrifuged sample of bacilli recognized by the bioconjugate. The concentrated pellet was allowed to dry to form a halo. The image

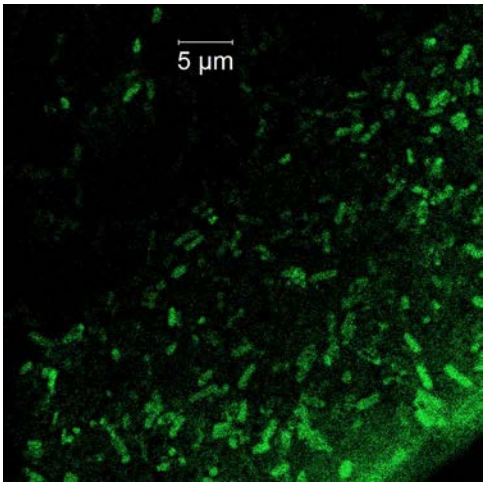

Figure 7. Dark-field of fluorescence emission of a centrifuged sample of bacilli recognized by the bioconjugate obtained in this work. A continuum emission of fluorescence due only to the bioconjugate was observed after the formation of a halo.

suggests that in addition to the observation of recognized bacilli, continuum emission of fluorescence due to the bioconjugate alone is also visible. This demonstrates the suitability of the nanoparticles of bioconjugate using the reported methodology for sensing.

Conclusion

A practical method to prepare a fluorescent bioconjugate based on gold nanoparticles for detection of *Salmonella* is reported. This bioconjugate was prepared by using protocols of conjugation of protein A on the surface of gold nanoparticles with subsequent functionalization over this layer using fluorescent antibodies. The obtained bioconjugate was used as probe to detect *Salmonella* by confocal microscopy in solution. This methodology may have the potential to develop a universal method for detecting different bacteria.

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