Esterase-Mediated Highly Fluorescent Gold Nanoclusters and Their Use in Ultrasensitive Detection of Mercury: Synthetic and Mechanistic Aspects

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ABSTRACT: The fast, accurate, and ultrasensitive detection of toxic mercury in real water samples is still challenging without the use of expensive sophisticated instruments. Herein, highly fluorescent gold nanoclusters (AuNCs) were synthesized using a newer protein templet, esterase (EST). The EST-AuNCs consisted of ∼25 Au atoms in the nanocluster having ∼2 nm size. EST-AuNCs were found to be highly stable in aqueous buffer with a wide range of pH (pH 4–12) and were also stable in powdered form. The fluorescence quantum yield of EST-AuNCs in deionized water was 6.2% which had increased to 7.8% upon the addition of 1 M NaCl (an increase of 23%). The EST-AuNCs selectively sense the toxic Hg²⁺ ions with higher sensitivity (limit of detection; 0.88 nM) with the linear range 1–30 nM. The test strips for rapid sensing of Hg²⁺ in real water samples were developed on the polymeric surface. The validation of sensing ability of EST-AuNCs suggested 94–98% recovery with linearity. Moreover, because of the widely reported applications of EST, the developed EST-AuNCs could also be used for another sensing, catalytic, and biomedical applications.

INTRODUCTION

Nanoclusters (NCs) have many remarkable characters compared with their larger one because of ultrasmall size (<2 nm) in sub-nanometer dimensions. The 3D arrangement of their free electrons gives rise to size-dependent electronic, optical, and chemical properties. Among all metal NCs, gold nanocluster is the most important one because of their high emission range suitable for electronic tissue penetration, biocompatibility, large Stokes shift, stability, ligand-dependent fluorescence properties. They also have a wide range of catalytic and biomedical applications. Various methods such as chemical reduction, template-assisted synthesis, etching, photo-reduction, and so forth to synthesize fluorescent gold NCs (AuNCs) have been widely reported. Among these procedures, a biosynthetic route is more advantageous in terms of easiness, environment friendliness, and speediness with good quantum yield. Recently, we have reported fluorescence gold nanoparticles using a plant extract having very low fluorescence quantum yield. Protein-directed synthesis of AuNCs is a single pot green synthetic route for the production of highly stable and highly fluorescent AuNCs. First, Xie et al. synthesized the highly fluorescent AuNCs using commercially available bovine serum albumin (BSA) and subsequently used them as a sensor mercury (Hg²⁺) with 0.5 nM limit of detection (LOD). Strong fluorescent property of metal NCs is one of the most important research areas in biosensing and bioimaging field. Quenching of fluorescence is the main mechanism behind the sensing of the trace amount of substances (i.e., various heavy metals, enzymes, etc.).

Hg²⁺ is a toxic heavy metal ion when binding to different cellular components leads to serious complications and death. Because of its higher toxicity in nanomolar concentration, there is a need for highly sensitive and selective sensing system to determine Hg²⁺ at very low concentration. Au⁺ can selectively sense Hg²⁺ ion by the interaction between the electrons present in “d” orbitals, hence, many systems containing Au⁺ ions have been reported as a sensing system for Hg²⁺. Chen et al. summarized most of them with LOD’s 0.5–5 nM. There is a major concern to prepare highly fluorescent AuNCs by a biological route having more selectivity and less LOD to sense the Hg²⁺ more precisely. Esterase (EST) is a class of enzymes extensively distributed in mammalian tissue. The enzyme is mainly responsible for the metabolism of xenobiotic; it catalyzes the hydrolysis of ester and amide containing compounds. Most of the ESTs contains a large number of amino acids (550) and have around 14 tyrosine, 10 tryptophan, 4 cysteines, 19 aspartates, 35 glutamate, and 16 methionine residues in its peptide chain. Tyrosine, tryptophan, and sulfur-containing amino acids are mainly responsible for the reduction of Au(III) ion in alkaline pH and the formation of gold nanocluster. Herein, we established a simple one-step EST-mediated synthesis of highly fluorescent AuNCs (EST-AuNCs), which can specifically and ultrasensitively detect Hg²⁺ (LOD = 0.88 nM). The
comparative properties of EST-AuNCs such as linear range and LOD with respect to the other similar sensors are furnished in the Table 1. A newer protein templet (EST) was initially used to synthesize the gold NCs. Before the final synthesis, the conditions for the synthesis of NCs were extensively optimized. The synthesized NCs were characterized using various techniques. The stability of EST-AuNCs in the various environmental conditions such as pHs, presence of organic chemicals, and salt concentrations was determined. Further, the test strips were developed to sense the Hg$^{2+}$ in real water samples. The sensing ability, selectivity, sensitivity, and mechanism of sensing were characterized using various techniques. Overall, highly fluorescent AuNCs were synthesized, characterized, and further developed as an ultrasensitive sensor of Hg$^{2+}$.

### RESULTS AND DISCUSSION

#### Synthesis of EST-AuNCs

Highly fluorescent AuNCs were synthesized using EST as a template for the reduction and stabilization of gold atoms in sub-nanometer sized clusters. The blank EST protein solution showed pale yellow color in visible light and emitted weak green fluorescence under UV at 365 nm because of the presence of aromatic amino acids (tyrosine, tryptophan, etc.). After 18 h of reaction at 35 °C, the slight color change was observed in the reaction mixture in visible light. Interestingly, the solution showed intense luminescence under UV (365 nm), because of ultrasmall NCs (<3 nm) having unique luminescence properties, indicating the formation of AuNCs (Figure 1a). The fluorescence excitation and emission wavelengths of EST-AuNCs were found to be 480 and 650 nm, respectively (Figure 1).

### Table 1. Comparative Properties of the Current Method for the Detection of Hg$^{2+}$

| material                  | synthesized using                   | real sample used                      | linear range | LOD     | refs |
|---------------------------|-------------------------------------|---------------------------------------|--------------|---------|------|
| AuNCs                     | BSA                                 | tap water                             | 1−20 nM      | 0.5 nM  | 11   |
| AuNCs                     | bacterial enoyl-ACP reductase        | tap water                             | –            | 50 μM   | 17   |
| AuNCs                     | chicken egg white                   | –                                     | 0.6−10 μM    | 0.51 μM | 18   |
| AuNCs                     | BSA                                 | river water                           | 0.2−60 μM    | 30 nM   | 19   |
| AuNCs                     | DNA                                 | human urine, lake water               | 0.1−100 μM   | 0.083 μM| 20   |
| AuNCs                     | BSA                                 | tap water, electroplating wastewater   | 25 nM to 5 μM| 8 nM   | 21   |
| AgNCs                     | sodium citrate, glutathione         | river water, tap water, blood serum   | 0.05−9 μM    | 12 nM   | 22   |
| AuNCs                     | dithioerythritol                    | lake water, tap water                 | 50−1000 nM   | 8.7 nM  | 23   |
| CD-AuNCs                  | BSA functionalized carbon dots      | tap water                             | 2−15 nM      | 0.73 nM | 24   |
| AuNCs                     | EST                                 | runnel water, tap water, water from water treatment plant | 1−30 nM    | 0.88 nM | proposed |

![Figure 1.](image.png)
The fluorescence quantum yield of EST-AuNCs (Q, $\sim$6.2%) in deionized water was calculated using relative quantum yield method$^{26}$ where rhodamine 6G was used as a standard (Q, 95%) (see Section S2). Further, the absence of plasmonic band (at 520 nm) in UV–vis spectra (Figure 1d) demonstrated the absence of larger nanoparticles in the solution which was confirmed by dynamic light scattering (DLS) and high-resolution transmission electron microscopy (HRTEM) analysis. The DLS spectrum and TEM analysis showed that the average NCs diameter was $<2$ nm (Figure 1e,f).

The MALDI TOF/TOF MS analysis of EST-AuNCs and respectively, which represented attribution of showed that the average NCs diameter was $<2$ nm (Figure (HRTEM) analysis. The DLS spectrum and TEM analysis synthesized NCs has been further con

The higher stability of 25-atom magic cluster has been most

The binding energy from the Au(0) [84.0 eV], con

The photoluminescence emission of EST-AuNCs (Figure 2). The photoluminescence properties of EST-AuNCs were checked in buffers of various different pHs with increasing salt concentration. No apparent change in photoluminescence was observed with a wide range of pHs (3.5–12) and phosphate buffer saline (10–100 mM, pH 7.4). Notably, the fluorescence intensity reduced slowly with the increase in pH more than 12, whereas an irreversible sudden decrease in the fluorescence was observed below pH 2.7. The study suggested that the fluorescence can be reversed by increasing its pH up to 11 for the EST-AuNCs solution and that pH had decreased up to 2.7. The fluorescence of the EST-AuNCs solution could not be reversed which has the pH below 2.7 (Figure S7). The initial decrease in fluorescence was due to the structural changes in the protein structures at lower pH, confirmed by circular dichroism (CD) spectra (Figures 3a,b, and S8). Moreover, dropping in the pH endorsed the zeta potential toward “zero”, which tends to the aggregation of NCs and responsible for the fluorescence quenching (Figure 3c). The initial decrease in fluorescence can be reversed upon increase in pH because the secondary structure can be restored to its initial stable state. Further decrease in the pH resulted the irreversible change in the secondary structure of the enzyme template, as well as complete aggregation of NCs endorsed the sudden decrease in the fluorescence. Here, the increased size of the NCs owes the main reason behind the fluorescence quenching as the photophysical properties of larger particles have different characteristics than small (<2 nm) NCs. The addition of salt in the solution of EST-AuNCs showed gradual increment in the fluorescence with respect to increase in the salt concentration (Figures 3d and S9). The fluorescence intensity of EST-AuNCs was increased by $\sim$23% at a higher salt concentration (1 M, NaCl). The higher salt concentration might have the favorable condition to stabilize the EST-AuNCs because of the structural stability of protein (Figure 3e). Moreover, increased ionic strength might have been responsible for the increased radiative decay of electrons through restriction of nonradiative electrons decay from the excited state to the ground state. However, the exact mechanism of this increased radiative decay needs more extensive study. The fluorescence quantum yield ($Q$) of EST-AuNCs in 1 M NaCl solution was found to be $\sim$7.8%. The stability of the EST-AuNCs in various other solvents (toluene, dimethyl sulfoxide, ethyl alcohol, methyl alcohol, isopropyl alcohol, diethyl ether, ethyl acetate, acetonitrile, and chloroform) was determined. The significant fluorescence quenching was observed with precipitation in all the solvents tested (Figure S10). This study suggested the instability of the EST-AuNCs in the tested solvents. The instability of the EST-protein in these solvents might be the most plausible reason of loss of the fluorescence.

Sensing of Hg$^{2+}$. The complete fluorescence quenching of EST-AuNCs (20 μM in aqueous solution) was observed within seconds upon the addition of 30 μM Hg$^{2+}$ (Figure 4a,b). Many

Figure 2. XPS spectra of EST-AuNCs. (a) Full spectrum showing binding energies of C, O, and S elements. (b) Au 4f XPS spectrum of EST-AuNCs. (c) C 1s spectrum of EST-AuNCs.
of the studies for Hg\(^{2+}\) sensing based on the AuNC fluorescence quenching showed diverse mechanistic aspects such as aggregation induced\(^{33,34}\) and AuNCs complex formation.\(^{35,36}\) Most recently, Senthamizhan et al. proposed the possible reason for highly selective AuNC fluorescence quenching, it might be the Au–Hg amalgam formation due to the enhanced interaction between Hg\(^{2+}\) and gold.\(^{37}\) These results also corroborate the findings reported by Xie et al. who suggested the interactions of Hg\(^{2+}\) with Au\(^{+}\) might be responsible for the selective fluorescence quenching of AuNCs.\(^{11}\) Here, we speculated that Au\(^{+}\) present on the surface of AuNCs could interact selectively to the Hg\(^{2+}\) and form Au–Hg amalgam. The Au–Hg interaction was confirmed by line EDS element mapping of the EST-AuNCs–Hg amalgam. The results showed that Hg was present with Au in the bonded state (Figure 4d). Here, mercury is uniformly located at the site of AuNCs confirming the strong interaction between mercury and gold. The XPS analysis of Hg-treated EST-AuNCs supported the formation of Au–Hg amalgam as the Hg 4f region has well separated (\(\Delta = 4.0\) eV), while no significant difference in other components (Au 4f and C 1s) of EST-AuNCs was observed in the XPS spectra (Figure 5a–d). The quenching effect was resulted because of the formation of d\(^{10}–d^{10}\) bonds because of the interaction between Au\(^{+}\) (4f\(^{14}5d^{10}\)) and Hg\(^{2+}\) (4f\(^{14}5d^{10}\)).\(^{17}\) Further, the size of Hg\(^{2+}\) treated EST-AuNCs was also \(\sim 2\) nm, which confirmed that very few Hg\(^{2+}\) ions are needed for fluorescence quenching (Figure 4c). Moreover, the fluorescence lifetime decay studies with different concentrations of Hg\(^{2+}\) (0, 5, 10, 30, and 50 nM) showed 0.22, 0.26, 0.22, 0.22, and 0.26 ns lifetime decay, respectively (Figure 5e). The study showed no significant change in the lifetime decay with respect to the blank revealed that, the quenching had resulted because of the static quenching or ground-state phenomenon.\(^{38}\) The substantial fluorescence quenching by Co\(^{2+}\) maybe because of the interaction between the enzyme and Co\(^{2+}\) ions. The same concentration of Hg\(^{2+}\) was having significant quenching efficiency (complete quenching) than Co\(^{2+}\). These results confirmed the sensitivity of AuNCs toward selective Hg\(^{2+}\) sensing.

Figure 3. (a) Relative fluorescence intensities of EST-AuNCs at different pHs, (b) CD spectra of EST-AuNCs at different pHs, (c) size distribution graphs of EST-AuNCs upon the addition of different amounts of 1 M HCl. The resulted solutions showed changed pHs 2.3, 2.1, and 1.9. Inset graph showing the zeta potential of EST-AuNCs at different pHs. (d) Relative fluorescence of EST-AuNCs at different salt concentrations, (e) CD spectra of EST-AuNCs in aqueous (black spectrum) and 1 M NaCl solution (blue spectrum).
In a typical experiment, various relevant metal ion salts (30 μM) were added to 20 μM aqueous solution of EST-AuNCs, and the fluorescence properties of the solution were analyzed. Despite the 100% fluorescence quenching upon the addition of Hg2+, no substantial change in the fluorescence intensity was observed with the various environmentally relevant metal ions, namely, Ca2+, Cd2+, Mg2+, Ni2+, Pb2+, Zn2+, Se4+, Cu2+, Al3+, Sn2+, Fe3+, Mn2+, and Co2+ (Figure 4a). The method is suitable to analyze the samples from different environmental sources because of the compatibility of EST-AuNCs in different buffers and pH. The interaction ability of Hg2+ with the Au+ determines the sensitivity of the AuNCs. The stronger interaction led to higher sensitivity, whereas weaker interaction indicates low sensitivity.11,39 Further, after the confirmation of selectivity by Hg2+ ions, it was important to determine the linearity and LOD. For this purpose, the lowest concentration of AuNCs (25 nM) was used. The higher concentration of AuNCs did not show the significant difference in the fluorescence upon addition of very low concentration of Hg2+. Thus, low concentrated EST-AuNCs solution (25 nM) was used for further study and determination of LOD and linearity. The effect of very small amount of Hg2+ could be easily tracked upon the fluorescence of 25 nM AuNCs solution using fluorescence spectrophotometer. After the addition of different concentrations (0.025–100 nm) of Hg2+ into the aqueous solutions of EST-AuNCs (25 nM), separately, the resulted solution showed the decrease in fluorescence intensities with the increase in Hg2+ concentration (Figures 4a–4d).

Figure 4. Estimation of sensing ability of EST-AuNCs for Hg2+ with respect to other ions. Characterization by fluorescence spectroscopy and UV–vis lamp. (a) Relative fluorescence intensities of 20 μM EST-AuNCs upon the addition of 30 μM different metal ion solutions (λex = 480 nm). (b) Upper and middle panels showing images of 20 μM EST-AuNCs solution with added 30 μM metal solutions in visible and UV light, respectively. The lower panel showing the image of EST-AuNCs test strips under UV after the treatment with 30 μM metal ions. Here, higher concentration (30 μM) of heavy metal ions were used for primary screening of selective quenching. (c) TEM images of EST-AuNCs–Hg at 50 and 5 nm scale. (d) Line EDS mapping of EST-AuNCs–Hg. The first image representing the scanning transmission electron microscopy image in which line EDS mapping was performed (marked). Other images showing the EDS map of carbon, oxygen, sulphur, gold, and mercury. Here, the aqueous solution of Hg2+ salt (30 μM) was added in the solution of EST-AuNCs (20 μM) and reacted for 30 min. The line EDS mapping was performed for the resulted reaction mixture.
More than 70 and 80% fluorescence quenching was observed upon the addition of 30 and 75 nM Hg$^{2+}$, respectively (Figure 6b). In the present curve, the inconsistent and nonlinear fall in fluorescence was observed with the addition of 0−1 nM Hg$^{2+}$, thus there was no quantitative relation between EST-AuNCs and Hg$^{2+}$ upon initial addition of Hg$^{2+}$. The linear decrease ($R^2 = 0.99$) was observed at 1−30 nM Hg$^{2+}$ which could be useful in the quantitative determination of Hg$^{2+}$ (Figure 6b inset). This linear quenching corroborates the phenomena of static quenching. Thus, the static quenching effect might be responsible for the linear fluorescence quenching. The fluorescence intensity decreased linearly with the addition of 1−30 nM of Hg$^{2+}$ (Figure 6b inset). Moreover, the estimated LOD to sense the Hg$^{2+}$ was 0.88 nM, (1.76 ppb) at a signal to noise ratio >3, was lower than the permitted level of Hg$^{2+}$ in the drinking water (2 ppb) as reported by the United States Environmental Protection Agency. The current method is highly sensitive and corroborated the methods reported in the literature using various fluorescent NCs (Table 1). Further, experiments were extended toward the development of paper test strip. Similar to the BSA stabilized AuNCs,$^{11,41}$ the EST-AuNCs were found to be stable at the polymeric support. For this purpose, the small pieces of Whatman paper no. 41 was soaked in EST-AuNCs solution (20 μM) for 24 h, rinsed with deionized water, and air-dried. The persistent fluorescence properties suggested that the developed strips could be stable for more than 6 months at 4 °C in an airtight container. The test strips were dipped into the 30 μM solution of different metal ions and various other compounds such as urea, uric acid, hydrogen peroxide, oxalic acid, and sodium sulphate. After 2 min, the strips were put out, air−dried, and observed under UV. All the strips showed fluorescence except the strip which was dipped into the solution of Hg$^{2+}$ (Figure 4b, panel 3). Apart from other metal ions and other tested compounds (Figure S12), Hg$^{2+}$ ions were selectively interacted with the Au$^+$ and were responsible for fluorescence quenching. Thus, these strips could be useful for the quick visual detection of Hg$^{2+}$ in the samples. Moreover, the recorded confocal laser scanning microscopy (CLSM)
images at various concentrations of Hg2+ showed the complete fluorescence quenching with 100 nM Hg2+ (Figure 7a). The CLSM images of a single strand of a test strip showed the complete fluorescence quenching within 35 min when treated with 100 nM Hg2+ (Figure 7b). The CLSM results suggested the application of the developed AuNCs in the real-time sensing of Hg2+.

**Analysis of Mercury in Real Water Samples.** To validate the sensing ability of the developed EST-AuNCs in real water samples, collected water samples were spiked with the Hg2+ (100 nM). The paper strips were treated with the water, and photos of each paper strip was captured at each 5 min interval. The results suggested that the samples which were spiked with the Hg2+ showed significant fluorescence inhibition, whereas samples without spiked concentration of Hg2+ did not have any effect on the fluorescence of the strips (Figure 8). These results also confirm that the other ions or chemicals present in the water from various sources do not have any effect on the fluorescence of the developed sensor. Thus, the results validated the stability of the developed sensor strips. Furthermore, the accuracy of the fluorescence quenching to determine the exact concentration in the real water samples was determined. The results suggested that the spiked water sample showed 94–98% accuracy with respect to the added amount of Hg2+. Further, to confirm the accuracy of the method, the quantitative inductively coupled plasma mass spectrometry (ICP-MS) was performed which showed 97–99% accurate quantity of added mercury (Table 2). Overall, the developed EST-AuNCs is mostly free from the effect of another component present in the real water sample from different sources and determines the accuracy and reliability of the present method for the determination of Hg2+ in real samples.

**CONCLUSION**

In conclusion, the red luminescent (Q, 7.8%) Au25NCs (<2 nm) using a new template (EST; a well-known protein) is successfully synthesized in a one-pot single-step method. The synthesized EST-AuNCs are highly stable in solution or in solid form at various pHs (4–12) as well as in higher salt concentration. The EST-AuNCs are employed in the highly selective and sensitive detection of Hg2+ (LOD, 0.88 nM). Moreover, the paper test strips are useful for the rapid visual detection of Hg2+ in the samples. Test strips are also useful for
real-time Hg\textsuperscript{2+} sensing using CLSM. The developed strips also showed their ability to sense the Hg\textsuperscript{2+} in the real water samples from different sources. The validation study confirmed the accuracy of sensing, more than 95%. Because of the unique conjugation and interaction properties of the protein/peptides, these NCs are not only were employed in the detection of Hg\textsuperscript{2+} but could be applicable for many other applications in material, biomedical, and catalysis science.

**EXPERIMENTAL SECTION**

**Materials.** Auriur chloride (AuCl\textsubscript{3}), EST from porcine liver, and rhodamine 6G were procured from Sigma-Aldrich (USA). Various metal ion salts were purchased from different vendors, that is, mercuric chloride, nickel(II) sulfate, zinc(II) acetate, and tin chloride from Sigma-Aldrich, USA; calcium chloride, cobalt chloride, sodium selenite, copper sulfate, aluminium sulfate, ferric chloride, and manganese chloride from Himedia, India; cadmium chloride from Qualigenes, India; magnesium chloride from, CDH, New Delhi, India; and lead(II) acetate from di

**Synthesis of Fluorescent EST-AuNCs.** Glassware’s were carefully washed with Aqua Regia, acetone, and deionized water. The reaction conditions were optimized for the synthesis of EST-AuNCs before the final synthesis. To synthesize EST-AuNCs, 10 mL of aqueous solution of AuCl\textsubscript{3} (6 mM) containing 1 mL 1 M NaOH was added dropwise to EST solution (15 mg/mL) under stirring at 600 rpm. After 2 min, the stirring rate was adjusted to 150 rpm and the reaction was allowed to proceed for 18 h at 35 °C. There was no obvious color change observed in visible light; however, it showed red fluorescence under UV at 365 nm. This solution was used for further characterization and stored at 4 °C for further use. EST-AuNCs were purified through dialysis (membrane cut-off: 10 kDa) in deionized water at 4 °C for 24 h to get rid of unreacted salt and NaOH. The purified AuNCs were lyophilized and stored at 4 °C for further characterization and use.

**Characterization.** The absorption and fluorescence spectra were recorded using Hitachi U-3900 UV–vis spectrophotometer and Varian carry-eclipse fluorescence spectrophotometer (Agilent), respectively. The fluorescence lifetime was recorded using Delafield modular fluorescence lifetime system (Horiba scientific). The particle size and zeta potential of EST-AuNCs were recorded using Zetasizer (Malvern Instruments, UK). The Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker) was used to analyze the molecular weights of EST and EST-AuNCs. An X-ray photoelectron spectrophotometer (XPS, PHI 5000 Versa Prob II, FEI Inc.) was used to determine the binding energy of gold and mercury atoms.

**Table 2. Validation of Current to Detect the Hg\textsuperscript{2+} in the Water Samples**

| s. no | standard addition of Hg\textsuperscript{2+} in deionized water (nM) | amount found in the spiked water sample |
|-------|---------------------------------------------------------------|---------------------------------------|
|       |                                                               | current method | ICP-MS |
|       |                                                               | nM    | %   | nM    | %   |
| 1     | 5                                                              | 4.75±0.31 | 95.04±6.26 | 4.85±0.44 | 97.04±8.90 |
| 2     | 10                                                             | 9.43±0.17 | 94.29±1.75 | 9.88±0.67 | 98.77±6.71 |
| 3     | 20                                                             | 19.57±0.34 | 97.87±1.67 | 19.99±0.64 | 99.96±3.21 |
| 4     | 30                                                             | 29.43±0.31 | 98.13±1.05 | 29.89±0.42 | 99.66±1.42 |

Functional groups of EST and EST-AuNCs were analyzed using an FTIR synthesis monitoring system equipped with an ATR and IR microscope (PerkinElmer). XRD spectra were recorded using a D8 Brucker XRD instrument. The thermal stability was characterized using differential scanning calorimetry (DSC 821e, Mettler Toledo). The structural changes in protein structure were determined using CD spectroscopy (Jasco J-815 CD spectrophotometer, USA). FEI Tecnai HRTEM fitted with EDS was used to perform TEM and line EDS mapping of elements.

**Detection of Hg\textsuperscript{2+} Using EST-AuNCs and Selectivity Study.** The solution of EST-AuNCs (20 μM) was prepared in 1 M NaCl salt solution, and the pH was adjusted to 7.2. The solution of different inorganic heavy metal salts (final concentration 30 μM; Hg\textsuperscript{2+}, Ca\textsuperscript{2+}, Cd\textsuperscript{2+}, Mg\textsuperscript{2+}, Ni\textsuperscript{2+}, Pb\textsuperscript{2+}, Zn\textsuperscript{2+}, Se\textsuperscript{4+}, Cu\textsuperscript{2+}, Al\textsuperscript{3+}, Sn\textsuperscript{2+}, Fe\textsuperscript{3+}, Mn\textsuperscript{2+}, Co\textsuperscript{3+}) was added to glass vials containing 200 μL solution of EST-AuNCs, separately. Each vial was then sonicated for 3 min and set aside for 30 min to complete the reaction. The solution in vials was then observed under UV at 365 nm. This study was performed for the initial screening and selectivity of fluorescence quenching in the presence of various salts. In another study, various other compounds (urea, oxalic acid, uric acid, hydrogen peroxide, and Na\textsubscript{2}SO\textsubscript{4}; 30 μM each) were added to the EST-AuNCs solution, and the effect on the fluorescence was determined. To determine the mercury sensing limit, the aqueous solution of EST-AuNCs (25 nM, pH 7.2) having 1 M NaCl, different concentrations of Hg\textsuperscript{2+} were added to make its final concentration (0.025–100 nM), separately (n = 6). The reaction mixtures were then sonicated for 3 min and set aside for 30 min to ensure the completion of the reaction. The fluorescence spectra were recorded at emission range 550–700 nm with λ\textsubscript{ex} 480 nm (slit width 20 each; excitation and emission). The relationship between the relative integrated intensities and Hg\textsuperscript{2+} concentration was plotted. The LOD and linear detection range of Hg\textsuperscript{2+} were determined.

**Development of Test Strips to Sense Hg\textsuperscript{2+}.** In the solution of EST-AuNCs (10 mL), small pieces (1 × 2 cm) of filter paper (41 no. Whatman) were soaked for 24 h. These papers were air-dried and stored at 4 °C for further use. To sense Hg\textsuperscript{2+}, 25 μL of Hg\textsuperscript{2+} solution of varied concentration was spotted on the separate strip and analyzed under UV (365 nm) after air drying. The color changes at various Hg\textsuperscript{2+} concentrations were observed. Test strips for CLSM were developed by soaking the filter strips into EST-AuNCs solution (50 nM) for 24 h and simultaneously washed in deionized water with shaking for 15 min. Completely air-dried strips were then treated with various concentrations of Hg\textsuperscript{2+} and analyzed under CLSM at excitation wavelength of 488 nm. The real-time sensing of Hg\textsuperscript{2+} was determined under CLSM using 100 nM Hg\textsuperscript{2+} by recording the images at a different time intervals.
Validation of the Sensing Ability Using Real Water Samples. Water samples from various sources (tap water from NIPER SAS Nagar, water from sewage treatment plant NIPER SAS Nagar, water from Chandigarh-Mohali city runnel) were collected. Water samples were initially centrifuged at 10 000g for 30 min, and the supernatant was collected. The supernatant was filtered through 0.22 μm filter tailored with the syringe filter assembly. The test strips were treated with the filtered water samples separately, and fluorescence of strips was observed under UV light (365 nm) and recorded up to 30 min. Further, in a separate experiment, water samples were spiked with Hg2+ (100 nM final concentration) before the centrifugation and filtration steps. After the centrifugation and filtration, the test strips were treated with the resulted water samples. The fluorescence of test strips was observed for 30 min under 365 nm UV light. Furthermore, to validate the ability of the method for the linear detection of the mercury in the real water samples, different concentrations of mercury solutions were prepared. The prepared solutions were mixed with the EST-AuNCs solutions separately up to 5, 10, 20, and 30 nM (final concentration) of Hg2+. The integrated fluorescence intensities of the resulted solution were recorded and the Hg2+ concentration was calculated using the standard linear equation of fluorescence quenching. The accuracy of the method was validated by measuring its concentration using ICP-MS (7700 series, Agilent Technologies).

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02505.

Supporting Information containing the optimization of the synthesis, procedure to determine the fluorescence quantum yield, and characterization data (PDF)

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Notes
The authors declare no competing financial interest.

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