Single Platform for Gene and Protein Expression Analyses Using Luminescent Gold Nanoclusters

Sunil Kumar Sailapu,† Deepanjalee Dutta,‡ Amarendra Kumar Sahoo,†∥ Siddhartha Sankar Ghosh,*†∥ and Arun Chattopadhyay*†∥

†Centre for Nanotechnology, ‡Department of Biosciences and Bioengineering, and ∥Department of Chemistry, Indian Institute of Technology Guwahati, Guwahati 781 039, Assam, India

Supporting Information

**ABSTRACT:** A single platform for gene and protein expression studies is proposed to pursue rapid diagnostics. A common method to synthesize gold (Au) nanoclusters on both DNA and protein template was developed using a benchtop device. The method of synthesis is rapid and versatile and can be applied to different classes of DNA/protein. Employing luminescent Au nanoclusters as the signal-generating agents, the device enables carrying out reverse transcriptase polymerase chain reaction and array-based analyses of multiple genes/proteins simultaneously using switchable holders and custom-designed software. The device and methods were applied to evaluate gene profiling related to apoptosis in HeLa cancer cells and further to analyze the protein expressions of glutathione-S-transferase (GST) and GST-tagged human granulocyte macrophage colony-stimulating factor (GST-hGMCSF) recombinant proteins purified from bacterial strains of BL21(DE3) Escherichia coli (E. coli). The device with user-friendly methods for diagnosis using the luminescence of Au nanoclusters offers potential use in disease diagnostics with a vision to extend health care facilities especially to remote geographical locations.

**INTRODUCTION**

Analyses of genes and proteins can be considered as two pillars key to the molecular basis of health analysis. An important aspect is to develop new methods and devices for the analyses so that not only faster and sensitive techniques are made available but also a broad spectrum of the global populace can accrue the benefit of such developments. This can possibly be achieved by combining the best of well-established conventional gene and protein analysis protocols and currently developing fields such as nanomaterials with extraordinary optical properties. Recent technological developments, in fields of genomics and proteomics, have helped to provide vital information coded in DNA and proteins to predict potential disease targets and mutations, to infer on disease susceptibility, and to discover novel therapeutic agents.1,2 The reverse transcriptase polymerase chain reaction (RT-PCR, in the case of genes) and high-throughput array-based methods (for analyses of genes and proteins) have been central to the achievements.3,4 However, the advancements have been associated with sophisticated instrumentation, complex and multistep fabrication and analysis techniques, and involved processes for functionalization of the probes to achieve high sensitivity and selectivity.5–8 Although an advanced technique such as quantitative PCR (qPCR) can be implemented for real-time sample analysis, its usage is not deemed necessary in many cases. Another technique, loop-mediated isothermal amplification though promising, suffers from drawbacks such as complicated primer designing method, challenges during multiplexing, contamination and cannot be used for the amplification of sequences of size >300 bp. Hence, semiquantitative PCR has remained a standard technique in molecular diagnostics. Some of the earlier reported devices (with thermocyclers and optical detection systems) were designed for performing real-time PCR (or quantitative RT-PCR) and are generally adopted for a limited number of samples (one to four) at a time.9,10 However, it is equally important to develop devices and easy methods to be applicable in routine usages where the semiquantitative PCR assay can be viewed as an useful alternative. A key aspect in PCR carried out by specific primers is detection of DNA amplicons through staining techniques for understanding the presence or absence of a particular gene. These semiquantitative PCR-based approaches could serve as a first-principle assay for understanding the infection status or the presence of a particular genetic material (as well as protein) for which qPCR is not mandatory. Additionally, there is concern over the use of hazardous organic dyes and radioactive isotopes which are less common but still considered for the staining of nucleic acids and proteins.11,12 Most of the organic dyes used as probes in staining techniques such as ethidium bromide (EtBr) were found to be cytotoxic and mutagenic. Other alternatives such as SYBRSafe, SafeView, RedSafe, and so forth, which are generally marketed as safe, were found to be able to permeate the cell membrane very rapidly and...
bind to DNA. They also show cytotoxicity and mutagenicity after metabolic activation. Importantly, current methods commonly use multiple probes (signal-generating agents) for the detection of both DNA and protein, thus lacking universality of approach leading to careful design optimizations of techniques and could be costly. Alternatively, one could develop new technology that would unify both the gene and protein analyses in a single platform by virtue of single biofriendly signal-generating probe to achieve rapid staining techniques. This would allow easy access of rapid diagnostics by a large population at an affordable cost.13,14

The efficiency of the PCR process has been improved through the usage of metal nanoparticles, quantum dots, carbon nanotubes, carbon nanoparticles, graphene, etc.15–18 The plasmonic19,20 and luminescent21 nanomaterials are useful as diagnostic probes in PCR and array-based methods. However, because of high sensitivity and background-related issues, fluorometric techniques are preferred over colorimetric assays.22 Use of conventional organic fluorophores is limited because of photobleaching, photoblinking, and often the carcinogenic nature of the molecule. Though quantum dots have been used as fluorometric tags in gene and protein expression studies as better materials, their toxicity is a concern.23–25 It is also worth

**Scheme 1.** (A) Layout of the Device and Schematic Illustrations of (B) Gene Expression Studies and (C) Protein Expression Studies

*For gene expression studies: (i) Amplification of DNA: genes of interest in the cDNA (cDNA converted from isolated RNA of cells) were amplified using the device. Synthesis of Au nanoclusters was then carried out on the PCR amplicon (in the tube) in a single temperature cycle using the thermocycler unit. The Au nanoclusters synthesized in the PCR products were imaged in the visualization unit under UV illumination. The intensities of emissions from the Au nanoclusters were analyzed using the software to generate information about amplification; (ii) DNA array: the PCR amplicons were heated and snap-cooled to obtain single-stranded products using the device and they were then spotted over the nitrocellulose membrane containing preimmobilized complementary ssDNA. Au nanoclusters were synthesized in a single temperature cycle on these spots and the membrane was imaged and analyzed in a similar way as described before. For protein expression studies: glutathione-S-transferase (GST) antigens were extracted and purified from *E. coli* BL21 (DE3) bacteria and were spotted on polyvinylidene difluoride (PVDF) membrane containing preimmobilized GST antibodies. Synthesis of Au nanoclusters was carried out on these spots using a single temperature cycle and the membrane was imaged and analyzed as detailed above. Here, NCs stand for nanoclusters.
noting here that most of the diagnostic techniques (for RT-PCR and microarray) adopt initial synthesis of nanomaterials followed by specific functionalization for conjugation with biomolecules. This particular approach demands extra labor (such as isolation of the probe) and processing and is thus time-consuming, making them not suitable for fast detection. Also, as interaction with biomolecules occurs post nanomaterials’ synthesis, there lies a possibility that their complete conjugation may be difficult to achieve because of potential loss of functionality to some extent. Validation in every step is necessary to certify the functionality, which is vital for subsequent interactions to occur. Alternatively, few atom luminescent metal nanoclusters are an exciting option as fluorophores because of their salient features including small size, high photostability, low toxicity with respect to other fluorophores, and low photoblinking. Recently, these metal nanoclusters have been applied as luminescent probes for various applications. Biomolecules such as DNA and proteins have been reported to be capable of directing the synthesis of fluorescent metal nanoclusters. Gold nanoclusters were also reported to be less cytotoxic and have good renal clearance as suggested by in vivo studies. However, extensive applications of these nanoclusters have so far been limited in rapid detection assays involving DNA and proteins because of the requirement of a large amount of precursors, a longer time period of synthesis, and extensive purification steps. In the current context, to employ them as signal-generating agents for both PCR and array-based techniques, the synthesis should be rapid, applicable for both DNA and protein (as direct template) in liquid as well as in solid phase. Provided such an efficient synthesis procedure is obtained integrating into the core functionality of PCR and array-based studies, it could pave for the development of a device with common mode of detection for the study of genes and proteins.

We report a single platform based approach for carrying out RT-PCR (nucleic acid amplification) and array-based analysis of multiple genes/proteins with luminescent Au nanoclusters as signal-generating agents using a benchtop device as illustrated in Scheme 1. The synthesis of Au nanoclusters (signal-generating probes) involved a rapid single-step method and can be carried out directly using DNA or protein. This synthesis of Au nanoclusters on the PCR products in the tube itself or on the hybridized PCR products in the nitrocellulose membrane or purified protein bound to an antibody attached to polyvinylidene difluoride (PVDF) membrane was achieved using the device itself and was all possible in one platform (Scheme 1). The synthesis was carried out in a rather short time to achieve nearly homogeneous Au nanoclusters without any need for further processing and thereby making it an excellent choice in applications as the diagnostic probe. The luminescence of the Au nanoclusters was proportional to the amount of the template present, that is, either DNA or protein concentration. Hence, study of the luminescence profile revealed the nature of reactions/interactions occurred provided there was a change in the amount of the biomolecule. This principle of detection was applied for the semiquantitative analysis of PCR products and for the array-based analysis of multiple samples of DNA and protein. In the case of PCR amplicon quantification, the device facilitated direct analysis of PCR products using the luminescence of the Au nanoclusters within the PCR tube, thus avoiding the additional step of gel electrophoresis. Paper-based membrane methods were adopted for array-based analysis to avoid complex fabrication procedure and made the whole process less expensive, easy to handle, flexible, and disposable so that the technique can easily be adopted for medical diagnostics even in remote areas. The traditional dot-blot assays are useful for the analysis of a small number of samples and are viable alternatives to highly sophisticated microarray platforms especially in the case of first-principle analysis. The ability to carry out membrane-based studies with rapid synthesis of Au nanoclusters using the device is an important element in the current study. To the best of our knowledge, the devices for qPCR with optical detection setup do not provide such a rapid detection platform for the membrane-based analysis of both genes and proteins using a single probe as demonstrated in the present work. Moreover, the current approach helps to avoid cross-contamination, which was observed in most of the devices based on microfluidics approach for qPCR.
The device and the methods proposed have been applied to evaluate gene profiling for the apoptotic pathway, where HeLa cancer cells were treated with a commercially available drug doxorubicin and their apoptotic gene expression profile was studied by Au nanocluster luminescence in an array-based method as illustrated in Scheme 1B and was compared with conventional EtBr staining. Also, for the study of protein expression, clinically important protein glutathione-S-transferase (GST) and GST-tagged human granulocyte macrophage colony-stimulating factor (GST-hGMCSF) expressed in Escherichia Coli (E. coli) BL21 (DE3) were used. The expressions were studied using array-based methods using the same device as shown in Scheme 1C, following purification and capture by an immobilized anti-GST antibody. The results matched with conventional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

RESULTS AND DISCUSSION

Benchtop Device Hardware and Software. The device is modular in design and constitutes two major blocks—thermocycler and visualization unit (Figures 1A and S1). The device is interfaced with computer/laptop and controlled through custom-developed software as shown in the Supporting Information, Figures S2 and S3. The software can operate all the blocks and allow entering device-related parameters, controlling the sequence of operations, performing image acquisition, and carrying out analysis. The thermocycler unit is used to achieve temperature cycling necessary for carrying out the RT-PCR process, gene/protein array-based experiments, and synthesis of Au nanoclusters on DNA/protein both in the liquid phase and on the membrane. This unit is equipped with switchable sample holders to accommodate both commercially available PCR tubes and the membrane (for array-based analysis), thus facilitating easy switching between the two processes as per need (Figure 1B). The bottom of the holder is exposed to Peltier unit (that is connected to heatsink and fan), which heats and cools the sample holder. The temperature of the holders can reach between 10 and 100 °C. For a PCR tube holder, the average ramp rate is about 0.95 °C/s (heating) and 1 °C/s (cooling). On the other hand, the average ramp rate for the membrane holder is about 1.2 °C/s (heating) and 1.3 °C/s (cooling). For the PCR process, a lid is placed on the top of the tube and is kept in hot condition to avoid condensation because of evaporation of the liquid. The temperature of the lid can quickly go up to 120 °C in about 150 s from room temperature (22 °C). Both the holders and the lid are equipped with sensors for monitoring the temperatures. A custom-designed-embedded circuit is the core electronic element responsible for the operation of the thermocycler. Au nanoclusters could be synthesized using the thermocycler unit itself with respective holders either inside the PCR tube (in the case of DNA) or on the membrane (in the case of gene/protein arrays). The visualization unit consists of a UV light source (254 nm), and the image, shown in Figure 2, was captured. It was observed that as the amount of the available template (end-point PCR amplicon) increases with the number.

PCR Amplification and Quantification Employing Au Nanoclusters. To evaluate the performance of the device toward DNA amplification, β-actin gene was amplified using specific primers—for 35 cycles. A similar set was amplified in a commercial PCR machine (Palm cycler), keeping the conditions of amplification same in both the cases. Standard gel electrophoresis with EtBr staining methods was employed to analyze the PCR amplicons. The gel images of the PCR amplicons as visualized in the gel documentation system are shown in the Supporting Information, Figure S4. Image analysis (densitometry) of gel bands revealed that a gene amplification efficacy of about 95% could be obtained using the device, in comparison to a commercial PCR machine. Instead of tedious gel electrophoresis and use of EtBr for quantifying PCR products,35 we devised a method to use luminescent Au nanoclusters to visualize the PCR amplicons following amplification in the device. We had earlier reported36 the development of a temperature-dependent facile and rapid synthesis of Au nanoclusters for DNA quantification. Adopting a single heating and cooling cycle-based synthesis (the condition similar to the conventional PCR) gives an advantage of synthesizing the Au nanoclusters probe alongside PCR for gene quantification. The current synthesis is found to be not affected by base pair compositions and sequence lengths.36 On the other hand, importantly, the luminescence of the formed Au nanoclusters was found to be affected only by the amount of dsDNA used for the synthesis, thus providing a method for semiquantification. Here, the same protocol was adopted with the flexibility to carry out the same in the PCR tube after gene amplification. The synthesis used the final PCR amplicon as the template with HAuCl₄ and 3-mercaptopropionic acid (MPA) as the reagents and was subjected to a single heating and cooling cycle (between the temperatures 95 and 15 °C) in the thermocycler unit. This resulted in the synthesis of Au nanoclusters in proportion to the amount of PCR amplicons (Supporting Information, Figure S5A). The Au nanoclusters exhibited luminescence with a peak at 585 nm, when excited by 254 nm light (Supporting Information, Figure S5B). PCR amplicons or DNA acted as a stabilizer by providing steric protection along with MPA (auxiliary small-molecule stabilizer) which passivates the Au nanoclusters through –S–Au bond linkages. It could be possible that the clusters were stabilized by both DNA and –COO– groups of MPA. In addition, MPA also contributed to the reduction of AuCl₄⁻ to produce nanoclusters.37 To employ these Au nanoclusters in quantification as signal-generating agents, β-actin gene was amplified for different cycles (e.g., 20, 25, 30, and 35) using specific primers in the thermocycler unit of the device. Then, Au nanoclusters were synthesized on the PCR amplicons by adding the reagents (HAuCl₄ and MPA) to the PCR tube, followed by heating to 95 °C for 2 min and then cooling down to 15 °C for 3 min. The PCR tubes were then directly imaged in the visualization unit under UV excitation of 254 nm, and the image, shown in Figure 2, was captured. It was observed that as the amount of the available template (end-point PCR amplicon) increases with the number.
of cycles of amplification, the formation of Au nanoclusters also increases monotonically, generating higher luminescence. Further, the luminescence of the synthesized Au nanoclusters provided a way for the semiquantification of the PCR products which is essential in ascertaining the presence/absence of a particular gene. It was also observed that the lowest amount of DNA that the synthesized Au nanoclusters could differentiate was for 20 cycles (starting with 0.5 μg of DNA). Another similar set was amplified for different cycles and validated by a standard process of agarose gel electrophoresis (with EtBr staining), which was in good agreement with the results obtained.

**Gene Expression Analysis.** These results encouraged us to extend the application of the device for studying the expression of multiple genes simultaneously in an array format, using these Au nanoclusters as the signal-generating agents. To achieve this, commercially obtained single-stranded oligonucleotide (β-actin) probes, in increasing concentrations, were immobilized in two rows on a nitrocellulose membrane. Commercially obtained complementary target strands were then hybridized on the spots in the second row only as shown in the Supporting Information, Figure S6. Au nanoclusters were then synthesized on all the spots in the membrane in the thermocycler unit as described above. Upon imaging in the visualization unit, the luminescence of the Au nanoclusters was evident in the spots of the membrane and it was observed that, for each concentration, the intensity in hybridized dsDNA spot was more in comparison to that in the control ssDNA spot, that is, for each of those spots present in the second row.

Commercially obtained sequences of common apoptotic genes (BCL-2, BAX, and Caspase-3) were chosen with the idea of extending the application of Au nanoclusters toward apoptotic gene expression profiling. Au nanoclusters were synthesized on different amounts of hybridized dsDNA for each set of genes. It was observed that, in each case, with the increase in the amount of the hybridized product, the luminescence increased proportionately, as shown in the Supporting Information, Figure S7. A set of experiments were

**Figure 2.** β-actin gene was amplified using specific primers using the present device—for 20, 25, 30, and 35 cycles. (i) Gel images corresponding to various cycles of EtBr-stained PCR products under UV illumination. (ii) Grayscale image of Au nanoclusters synthesized on different cycles of PCR amplicons under UV excitation (254 nm).

**Figure 3.** Study of expression of multiple genes in control HeLa cells and doxorubicin-treated HeLa cells. (A), (B) Grayscale images under UV illumination (254 nm) of Au nanoclusters synthesized on various genes (spots) in control and treated HeLa cells. First, commercially obtained ssDNA (as the capture agent) of BCL-2, BAX, and Caspase-3 in increasing amounts ((i) 0.18, (ii) 0.37, and (iii) 0.74 μg; indicated by the arrow) were immobilized on the nitrocellulose membrane in a single row. Following this, increasing amounts of PCR amplicons of BCL-2, BAX, and Caspase-3 for (i) 0.18, (ii) 0.37, and (iii) 0.74 μg obtained from control and doxorubicin-treated cells were heated and snap-cooled to achieve single-stranded products and were subsequently hybridized to the above-mentioned capture probes in the specific row, respectively. Au nanoclusters were then synthesized on all the spots of the membrane. (C) Relative luminescence intensity of Au nanoclusters synthesized on BCL-2, BAX, and Caspase-3-hybridized dsDNA in control and treated HeLa cells (obtained following the image analysis). The graph depicts the averaged intensity of data (relative to β-actin gene) from three separate experiments with their standard deviation. (D) Gel electrophoresis with EtBr staining of PCR amplicons of BCL-2, BAX, and Caspase-3 from control and treated HeLa cells. L1 and L2 lanes correspond to control and treated samples. Experiments were done in triplicates, *P < 0.05 with respect to control, **P < 0.01 with respect to control, and ***P < 0.001 with respect to control.
also performed with Au nanoclusters synthesized on non-complementary pairs of commercially obtained sequences. The analysis showed that the luminescence intensity of non-complementary pairs was similar to that of ssDNA controls immobilized on the membrane, as shown in the Supporting Information, Figure S8, possibly because of washing away of the noncomplementary strands. These results suggest that the luminescent Au nanoclusters are a viable alternative to commonly used organic dyes for studying gene expression.

For the application of Au nanoclusters in the study of relative gene expressions during apoptosis in cancer cells, in vitro, relative gene expression studies were carried out in HeLa cells, which were subjected to apoptosis via anticancer drug doxorubicin. The apoptosis specific genes in control and doxorubicin-treated cells (BCL-2, BAX, and Caspase-3) were then amplified, in the device, using gene-specific primers. Commercially obtained corresponding ssDNA apoptotic marker genes were immobilized on the nitrocellulose membrane in increasing amounts per spot. After this, PCR amplicons were heated and then snap-cooled in the thermocycler (to obtain single-stranded products), and they were subsequently transferred to the above spots containing ssDNA. Following hybridization, Au nanoclusters were synthesized on these spots, and the gene regulation outputs were quantified in correlation to the luminescence profile. As is evident from the relative intensities in Figure 3, the apoptotic genes BAX and Caspase-3 were upregulated and BCL-2 was downregulated with respect to endogenous control β-actin, signifying apoptosis in the case of doxorubicin-treated HeLa cells in comparison to that in the control HeLa cells. Here, we would like to mention that there is an apparent anomaly in the observed data related to the concentration of 0.74 μg of BCL-2, which is expected to result in the highest luminescence among the set. This is caused because of the possible saturation of the luminescence at concentration above 0.37 μg, and any further increase in concentration caused a decrease in luminescence intensity, as observed at 0.74 μg. However, this does not affect the primary aim to understand the difference between the control and treated samples and could be well-demarcated (at 0.74 μg) irrespective of the decrease in luminescence. Conventional EtBr gel electrophoresis studies corroborated the aforementioned results obtained by the use of Au nanoclusters. Hence, Au nanoclusters were successfully applied to probe the expressions of multiple genes governing the apoptosis pathway in the HeLa cells. The short time and minimum precursor requirement compared to earlier reports make the Au nanocluster probe ideal for point-of-care detection. In addition, the current method did not form any larger-sized Au nanoparticles alongside Au nanoclusters, which was generally observed in previously reported works. Also, the current method was applicable for the synthesis of Au nanoclusters on both linear and plasmid forms of DNA. To the best of our knowledge, there is no report of a common method for the formation of Au nanoclusters on both linear and plasmid forms. These Au nanoclusters were also found to be biocompatible and nontoxic.

**Rapid Synthesis of Au Nanoclusters with Proteins.** Building on the successful applications of Au nanoclusters in assaying DNA, we developed a novel method of synthesizing Au nanoclusters on protein templates. This was to combine protein expression studies alongside gene analysis in the same device. The synthesis in the presence of proteins also involved a single-step heating and cooling cycle with similar precursors as in the case of DNA. Au nanoclusters were synthesized on bovine serum albumin (BSA) as the template—using HAuCl₄ and MPA as the reagents—by heating to 95 °C for 2 min and cooling to 15 °C for 3 min. Au nanoclusters thus formed emitted luminescence at 880 nm, when excited by 300 nm light. The formation of the nanoclusters was found to be dependent on the amount of protein. Also, the nanocluster synthesis is not specific to particular protein sequence, and this has been demonstrated using different classes of proteins. The versatility makes this assay applicable to a wide range of proteins, which is necessary for diagnostic applications (Supporting Information, Figure S9).

The luminescence profile of the Au nanoclusters for a range of concentrations of protein (BSA) and HAuCl₄ is shown in the Supporting Information, Figure S10. The surface intensity plot (Supporting Information, Figure S10) indicated that the luminescence intensity increased with the amount of HAuCl₄ (or protein) for a range of concentrations of either protein (or HAuCl₄), followed by decrease in the luminescence after a certain concentration. As in the above case with PCR amplicons/DNA, proteins are also a well-known template for nanocluster synthesis. Hence, similar to the nanocluster synthesis using DNA, here protein acted as the stabilizer with MPA as a reducing agent. The fluorescence spectrum of only MPA and HAuCl₄ showed the formation of extremely low-intensity Au nanoclusters, while no formation of Au nanoclusters was found in the case of only protein and HAuCl₄. However, in the presence of protein along with MPA and HAuCl₄, the intensity of the formed Au nanoclusters was significantly high and was also found to be stable in nature. Transmission electron microscopy (TEM) showed the formation of small particles owing to Au nanoclusters and also indicated an increase in the particle size from 0.75 ± 0.27 to 5.81 ± 2.05 nm, with increasing concentrations of HAuCl₄ (Supporting Information, Figure S11). Also, circular dichroism (CD) spectroscopy revealed that the formation of Au nanoclusters did not alter significantly the 3D structure of BSA (Supporting Information, Figure S12). The photoluminescence quantum yield of Au nanoclusters on proteins was found to be 4.5%, thus making them suitable for sensing applications. Also, when compared to an organic dye rhodamine 6G with the fluorescence decrease rate of 2.7% per min, the Au nanoclusters showed a fluorescence decrease rate of 0.26% per min, indicating higher photostability (Supporting Information, Figure S13). The matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis (Supporting Information, Figure S14) showed a distinct peak at 71171 (m/z). The m/z difference between BSA and Au nanoclusters is 4350.8 (m/z), favoring possible formation of (AuŒ16(MPA)œ1 + 2Na⁺ − H⁻)−3 corresponding to a metal cluster with 16 Au atoms.

**Protein Expression Analysis.** For the application of these Au nanoclusters in protein expression studies, the expression profiling of GST and GST-hGMCSF in E. coli BL 21 (DE3) cells was performed after their isolation and purification. The integrity and functionality of the isolated proteins (GST and GST-hGMCSF) were analyzed by using standard SDS-PAGE, CD spectroscopy, Bradford assay, and 1-chloro-2,4-dinitrobenzene (CDNB) assay (Supporting Information, Figures S15 and S16). In SDS-PAGE, bands corresponding to GST and GST-hGMCSF were observed at 26 and 42 kDa, respectively, and concentrations of the proteins obtained from the Bradford assay were 239 and 96 μg/mL for GST and GST-hGMCSF, respectively. CD spectroscopy confirmed that 3D conformation of GST as well as GST-hGMCSF was intact for functionality. Also, the CDNB assay specific for the determination of the GST enzyme activity confirmed that the GST was functional for both the cases of GST and GST-hGMCSF.
After obtaining the proteins (GST and GST-hGMCSF) in their functional forms, they were allowed to interact with their respective anti-GST antibodies previously immobilized on the PVDF membrane along with their GST, GST-hGMCSF, and anti-GST antibody controls. Then, Au nanoclusters were synthesized on the respective spots using the thermocycler unit in a single cycle of heating and cooling as in the case of DNA (described earlier). Thereafter, the membrane was imaged using the visualization unit, and the luminescence intensity of the Au nanoclusters was found to be the highest in the case of GST−anti-GST antibody conjugate followed by GST-hGMCSF−anti-GST antibody conjugate, when compared to only GST, GST-hGMCSF, and anti-GST antibody as shown in Figure 4. These results corroborated the standard SDS-PAGE and Bradford assay results obtained previously. As the formation of the nanoclusters was found to be dependent on the amount of protein, it was observed that in the case of immobilized antibodies, or only GST protein on the membrane, there was no visible formation of nanoclusters as an adequate amount of protein was absent in both these cases. Whereas when both the immobilized antibodies along with the GST protein were present together, the increase in the amount of the protein content led to the formation of nanoclusters. Hence, in the absence of the analyte, which can be either the antibody or the antigen depending on the assay, the luminescence will not be generated or will be always less compared to the antigen—antibody conjugate. As a control experiment, specific GST protein and a nonspecific BSA protein interacted with the anti-GST antibody, and it was found that the luminescence did not get enhanced in the case of BSA as it did with increasing concentrations of GST, possibly because of washing away of the nonspecific BSA antigen (Supporting Information, Figure S17). Hence, it can be concluded that the luminescent Au nanoclusters, synthesized in situ, were efficient probes for the protein expression studies. The same advantages as mentioned above for the use of Au nanoclusters as probes for gene expression studies also apply for the protein expression studies. The rapid method of synthesis directly on PCR products/proteins with minimum precursor concentrations, biocompatibility, and concentration-dependent luminescence makes the current study an important addition in the analysis of gene/protein expression.

Enhancement of Sensitivity with Zinc Ions. It may further be mentioned here that in array-based analysis, involving either gene or protein, addition of zinc ions during the synthesis of Au nanoclusters enhanced the luminescence intensity of the Au nanoclusters. As shown in the Supporting Information, Figure S18, in one of the rows, zinc ions were added during the synthesis of Au nanoclusters. As is evident from the image analysis, the luminescence intensity increased in the case where zinc ions were added to the reaction mixture. The reason for the increase in luminescence may be attributed to the aggregation of Au nanoclusters caused by zinc ions. Thus, this technique can be used for the enhancement of luminescence in the case of lower signal intensity.

CONCLUSIONS

We presented a single platform for the assaying of genes and proteins based on luminescent biofriendly Au nanoclusters using...
a benchtop device. The facile and rapid synthesis of biocompatible signal-generating agents (Au nanoclusters) on both DNA and proteins allowed semiquantitative and qualitative analyses. Further, it provided a method of synthesis of Au nanoclusters as a common probe for both DNA and protein studies (in liquid as well as samples on the membrane), PCR amplicon detection, and membrane-based studies in a single device. The device was able to deliver PCR amplification efficiency of about 95% in comparison to the commercially available machine. The custom-developed software simplified the operation to enhance the user experience and to perform tasks like providing input parameters and image acquisition and analysis. The modular design of the device permits easy upgradation. Importantly, the techniques, methods, and materials employed are bio- and environmentally friendly. Taken into account all these sublime features, the device and the integrated methods may add a new dimension to the existing techniques with the amalgamation of nanotechnology and biology. This may create motivation to converge various techniques and develop methods in a view to make rapid diagnostics available to a majority of people across the globe.

**METHODS**

**Amplification of DNA with the Device.** β-actin gene was amplified for 35 cycles using specific primers in both the conventional PCR machine (Palm cycler) and the present device, keeping the conditions as initial denaturation at 95 °C for 3 min followed by 35 cycles of three phases: 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. After this, final extension was carried out at 72 °C for 10 min. The PCR amplicons obtained from both the devices were analyzed using standard gel electrophoresis with EtBr staining. The stained gel was visualized under UV illumination as shown in the Supporting Information, Figure S4. The amplification achieved by the present device was about 95% compared to conventional PCR machine as analyzed from the image.

**Quantification of PCR Products Using Au Nanoclusters.** Two sets of β-actin gene were amplified for 20, 25, 30, and 35 cycles using specific primers using the thermocycler unit of the device with the conditions as initial denaturation at 95 °C for 3 min followed by the specific number of thermal cycles with each cycle as 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and final extension for 10 min.

In the first set, Au nanoclusters were synthesized using the thermocycler with the following protocol. HAuCl₄ (1.0 mM, 17 wt% Au, in dilute HCl; 99.99%, Sigma-Aldrich) and 0.01 M MPA (Sigma-Aldrich) were added in 3:1 (v/v) ratio to the end-point PCR product (inside the PCR tube itself), heated at 95 °C for 2 min, and then cooled at 15 °C for 3 min. This resulted in the formation of Au nanoclusters in proportion to the amplification. The final PCR tubes containing the PCR amplicons with synthesized Au nanoclusters were then imaged and analyzed using the visualization unit under UV excitation of 254 nm. Standard gel electrophoresis with EtBr staining is carried out for the second set of PCR amplicons. The gel was visualized under UV illumination.

**Gene Expression Studies.**

a) cDNA extraction from control HeLa cells and doxorubicin-treated HeLa cells: HeLa cancer cells were cultured in two 60 mm culture plates with a cell density of 1 × 10⁶ cells. Keeping one of the plates as the control, the other plate was treated with anticancer drug doxorubicin for 24 h. Using the standard RNA protocol, RNA was isolated from both the cells (control and treated). CDNA was obtained from mRNA using the thermocycler (at 42 °C for 40 min and 95 °C for 2 min) with Verso cDNA kit.

b) Amplification of specific genes using the thermocycler unit of the device: BAX, BCL-2, and Caspase-3 along with endogenous control β-actin were amplified using specific primers for 35 cycles (denaturation: 95 °C for 3 min; 35 cycles with three phases: 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and final extension for 10 min).

c) Immobilization of complementary oligonucleotide on the nitrocellulose membrane: nitrocellulose membrane (Zeta Probe blotting membranes; cut to maximum dimensions of 40 mm × 40 mm) was activated in 1X SSC (saline sodium citrate) buffer and was allowed to air-dry. The commercial oligonucleotides were spotted and UV-cross linked in an array format by the standard process.

d) Hybridization of heated and snap-cooled PCR products to the complementary oligonucleotides immobilized on the nitrocellulose membrane: the PCR products were heated and snap-cooled and hybridized to their respective immobilized complementary nucleotides by the following process.

The membrane was blocked before hybridization using blocking solution (1% PVP-10, 1% PEG 6000, and 0.05% Tween-20) for 15 min to avoid unspecific binding. The hybridization was carried out in 5X SSC buffer and 10% poly(ethylene glycol) (PEG) 6000 at 60 °C for half an hour. The membrane was then washed with 1X SSC buffer.

e) Synthesis of Au nanoclusters on a hybridized membrane: after hybridization, synthesis of Au nanoclusters was carried out on the spots by adding 1.5 μL of 0.7 mM HAuCl₄ and 0.5 μL of 0.01 M MPA, followed by heating the membrane using the thermocycler at 95 °C for 2 min and then cooling at 15 °C for 3 min.

f) Image acquisition and analysis: the membrane with synthesized Au nanoclusters was imaged and analyzed using the visualization unit using custom-developed software under UV illumination (254 nm).

**Synthesis of the Au Nanoclusters Using BSA as the Template.** For synthesis, 20 μL of (0.05–1.0 mg/mL) BSA (Himedia) was taken, and to that 0.4 μL of 10 mM HAuCl₄ and 0.16 μL of 0.11 M of MPA were added, and the mixture was heated at 95 °C for 2 min and then cooled at 15 °C for 3 min.

**Protein Expression Studies.**

a) Expression and purification of GST and GST-hGMCSF in *Escherichia coli* BL21 DE3: for primary culture, 20 μL of *E. coli* BL21 DE3 stock harboring pGEX4t2 vector was inoculated in 3 mL of LB (Luria–Bertani) media with 3 μL of 100 mg/mL ampicillin and was incubated overnight at 37 °C (180 rpm). The grown primary culture (2 mL) was inoculated into 200 mL LB media with 200 μL of 100 mg/mL ampicillin and was incubated at 37 °C (180 rpm) until an optical density (O.D.) of 0.6 was obtained. Following this, induction was given by 1 mL of isopropyl-β-D-thiogalactopyranoside (24 mg/mL) at 24 °C (180 rpm, 6 h). The cells were then centrifuged at 6000 rpm (4 °C, 7 min), and the pellet hence obtained was stored at −20 °C.

For the preparation of lysis buffer (7 mL), 100 μL of 1 mM ethylenediaminetetraacetic acid and 100 μL of 1 mM phenylmethane sulfonyl fluoride were added to 1X...
phosphate-buffered saline (PBS). The cell pellet was then resuspended in lysis buffer homogenously and was sonicated with a probe sonicator for 5 min. Centrifugation was carried out at 12,000 rpm (4 °C, 20 min), and the supernatant was collected. The supernatant obtained in the previous step contained solubilized protein. It was first filtered through a 0.45 μm syringe filter and then put into a glutathione agarose beads column (prepared previously by the standard method) for half an hour. Flow-through fractions were collected, followed by washing of the column eight times with PBS. For elution buffer preparation, 20 mg of reduced glutathione was added to 5 mL of 50 mM tris (pH 8). Recombinant GST bound to the affinity column was eluted with the elution buffer after 20 min. Multiple-flow through fractions were collected and analyzed using 12% SDS PAGE. A similar protocol was adopted for the isolation and purification of GST-hGMCSF.14
b) Estimation of protein by the Bradford assay: the concentration of the purified recombinant GST proteins was estimated using the Bradford assay. The standard solution was prepared using the BSA of concentrations 0.5, 1, 2, 4, and 10 μg/mL, and 10 μL of the protein sample (recombinant GST proteins) was used for analysis along with 90 μL of Bradford reagent solution (Sigma-Aldrich). It was allowed to react at room temperature for 10 min in dark, and the optical density at 595 nm was measured using a TECAN Elisa plate reader.
c) Enzyme activity: GST activity was determined using the CDNB assay: the increasing amount of protein solutions with 1X PBS buffer (pH 7.5), 1.0 mM GSH, and 1.0 mM CDNB, amounting to the total volume of 100 μL, was taken in a 96-well plate. The control wells contained PBS, CDNB, and glutathione. After 10 min, the change in absorbance with respect to control was measured at 340 nm using a Tecan Elisa plate reader. One unit of activity is defined as the formation of 1 μM product per min at 25 °C ($\varepsilon_{340}$ is the extinction coefficient at 340 nm in a 96-well plate is 53 for CDNB, and $D$ is the dilution factor).

\[
\text{Change in absorbance (} \Delta A_{340} / \text{min} = \frac{A_{340, \text{final}} - A_{340, \text{initial}}}{\text{reaction time}} \\
\text{GST activity (} \mu \text{mol/mL/min}) = \frac{(\Delta A_{340} / \text{min}) \times V(\text{mL}) \times D}{V_{\text{ext}}(\text{mL}) \times \varepsilon_{340}}
\]
d) Immobilization of primary antibody on the PVDF membrane in an array pattern: the primary antibody specific to the GST antigen was immobilized on the PVDF membrane (IMMOBILON P 0.45 μm membrane) by spotting different dilutions of primary antibody after activating the membrane of suitable dimensions (with maximum dimensions of 40 mm × 40 mm) in methanol. After spotting, the membrane was allowed to air-dry for 15–20 min.
e) Interaction of antigens with antibodies on the PVDF membrane: the membrane was blocked using blocking solution (as mentioned above) for 30 min to avoid unspecific binding, after that the membrane was incubated with respective GST antigens for 30 min and was washed with PBST (PBS with Tween 20) buffer for reducing nonspecificity.
f) Synthesis of Au nanoclusters on the PVDF membrane: after antigen–antibody interactions, Au nanoclusters were synthesized on the spots, by adding 1.5 μL of 0.7 mM HAuCl₄ and 0.5 μL of 0.01 M MPA followed by heating the membrane using a thermocycler at 95 °C for 2 min and then cooling at 15 °C for 3 min.
g) Image acquisition and analysis: the membrane with synthesized Au nanoclusters was imaged and analyzed using the visualization unit using custom-developed software under UV illumination (254 nm).

### Statistical Analysis
Data were expressed as mean value ± standard deviation (SD). Student’s t-test was employed to test significant differences between the experimental groups. (*P < 0.05, **P < 0.01, ***P < 0.001).

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b01739.

- TEM, emission spectra, software snapshots, quantum yield, photostability, MALDI, CD spectroscopy, Bradford assay, enzyme activity assay, and membrane-based analysis (PDF)

## AUTHOR INFORMATION

### Corresponding Authors
*E-mail: sghosh@iitg.ernet.in (S.S.G.).
*E-mail: arun@iitg.ernet.in (A.C.).

### ORCID

- Sunil Kumar Sailapu: 0000-0002-0923-0879
- Deepanjalee Dutta: 0000-0002-4695-5262
- Siddhartha Sankar Ghosh: 0000-0002-7121-5610
- Arun Chattopadhyay: 0000-0001-5095-6463

### Present Address
- Assistant Professor, Indian Institute of Information Technology, Allahabad, Uttar Pradesh 211012, India (A.M.S.).

### Author Contributions

S.K.S contributed ideas and developed the device, designed experiments, analysed results. D.D. contributed ideas, designed and carried out experiments, and analyzed results. A.K.S. contributed ideas. S.S.G contributed ideas and helped in analyses. A.C. conceived the idea and helped implement them. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

### Funding

We thank the Department of Electronics and Information Technology (no. 5(9)/2012-NANO (Vol. II)), Government of India for financial support.

### Notes

The authors declare the following competing financial interest(s): an Indian as well as international patent application has been filed based on the content of the manuscript.

### REFERENCES

1. Lockhart, D. J.; Winzeler, E. A. Genomics, Gene Expression and DNA Arrays. *Nature* 2000, *405*, 827–836.
2. Tyers, M.; Mann, M. From Genomics to Proteomics. *Nature* 2003, *422*, 193–197.
Facilitates HER2 Testing of Breast and Gastric Carcinomas. Genes Nutr. 2008, 3, 153–157.

Brandt, S.; Klokstra, S.; Altmann, T.; Kehr, J. Using Array Hybridization to Monitor Gene Expression at the Single Cell Level. J. Exp. Bot. 2002, 53, 2315–2323.

Quijada, L.; Soto, M.; Requena, J. M. Genomic DNA Macrorrays as a Tool for Analysis of Gene Expression in Leishmania. Exp. Parasitol. 2005, 111, 64–70.

Randolph, J. B.; Waggoner, A. S. Stability, Specificity, and Fluorescence Brightness of Multiply-Labeled Fluorescent DNA Probes. Nucleic Acids Res. 1997, 25, 2923–2929.

Moreira, B. G.; You, Y.; Owczarzy, R. Cy3 and Cy5 Dyes Attached to Oligonucleotide Terminus Stabilize DNA Duplexes: Predictive Thermodynamic Model. Biochem. Physiol. 2015, 198, 36–44.

Toutchkine, A.; Nalbant, P.; Hahn, K. M. Facile Synthesis of Thiophene- Reactive Cy3 and Cy5 Derivatives with Enhanced Water Solubility. Bioconjugate Chem. 2002, 13, 387–391.

Ahberg, C. D.; Ilic, B. R.; Manz, A.; Neužil, P. Handheld real-time PCR device. Lab Chip 2016, 16, 586–592.

Mulberry, G.; White, K. A.; Vaidya, M.; Sugaya, K.; Kim, B. N. 3D printing and milling a real-time PCR device for infectious disease diagnostics. PLoS One 2017, 12, no. e0179133.

Espina, V.; Woodhouse, E. C.; Wulfkuhle, J.; Asmussen, H. D.; Petricoin, E. F.; 3rd; Liotta, L. A. Probing the Nanoscale: Using Amino-Modified Silica-Coated Magnetic Nanoparticles as a Main Model. Bioconjugate Chem. 2011; pp 359–365.

Moreira, B. G.; Owczarzy, R. Cy3 and Cy5 Dyes Attached to Oligonucleotide Terminus Stabilize DNA Duplexes: Predictive Thermodynamic Model. Biochem. Physiol. 2015, 198, 36–44.

Toutchkine, A.; Nalbant, P.; Hahn, K. M. Facile Synthesis of Thiophene- Reactive Cy3 and Cy5 Derivatives with Enhanced Water Solubility. Bioconjugate Chem. 2002, 13, 387–391.

Ahberg, C. D.; Ilic, B. R.; Manz, A.; Neužil, P. Handheld real-time PCR device. Lab Chip 2016, 16, 586–592.

Mulberry, G.; White, K. A.; Vaidya, M.; Sugaya, K.; Kim, B. N. 3D printing and milling a real-time PCR device for infectious disease diagnostics. PLoS One 2017, 12, no. e0179133.

Espina, V.; Woodhouse, E. C.; Wulfkuhle, J.; Asmussen, H. D.; Petricoin, E. F.; 3rd; Liotta, L. A. Probing the Nanoscale: Using Amino-Modified Silica-Coated Magnetic Nanoparticles as a Main Model. Bioconjugate Chem. 2011; pp 359–365.

(3) Gaj, S.; Eijssen, L.; Mensink, R. P.; Evelo, C. T. A. Validating Nutrient-Related Gene Expression Changes from Microarrays Using RT-PCR Arrays. Genes Nutr. 2008, 3, 153–157.

(4) Brandt, S.; Klokstra, S.; Altmann, T.; Kehr, J. Using Array Hybridization to Monitor Gene Expression at the Single Cell Level. J. Exp. Bot. 2002, 53, 2315–2323.

(5) Quijada, L.; Soto, M.; Requena, J. M. Genomic DNA Macrorrays as a Tool for Analysis of Gene Expression in Leishmania. Exp. Parasitol. 2005, 111, 64–70.

(6) Randolph, J. B.; Waggoner, A. S. Stability, Specificity, and Fluorescence Brightness of Multiply-Labeled Fluorescent DNA Probes. Nucleic Acids Res. 1997, 25, 2923–2929.

(7) Moreira, B. G.; You, Y.; Owczarzy, R. Cy3 and Cy5 Dyes Attached to Oligonucleotide Terminus Stabilize DNA Duplexes: Predictive Thermodynamic Model. Biochem. Physiol. 2015, 198, 36–44.

(8) Toutchkine, A.; Nalbant, P.; Hahn, K. M. Facile Synthesis of Thiophene-Reactive Cy3 and Cy5 Derivatives with Enhanced Water Solubility. Bioconjugate Chem. 2002, 13, 387–391.

(9) Ahberg, C. D.; Ilic, B. R.; Manz, A.; Neužil, P. Handheld real-time PCR device. Lab Chip 2016, 16, 586–592.

(10) Mulberry, G.; White, K. A.; Vaidya, M.; Sugaya, K.; Kim, B. N. 3D printing and milling a real-time PCR device for infectious disease diagnostics. PLoS One 2017, 12, no. e0179133.

(11) Espina, V.; Woodhouse, E. C.; Wulfkuhle, J.; Asmussen, H. D.; Petricoin, E. F.; 3rd; Liotta, L. A. Probing the Nanoscale: Using Amino-Modified Silica-Coated Magnetic Nanoparticles as a Main Model. Bioconjugate Chem. 2011; pp 359–365.
(42) Chaubey, N.; Ghosh, S. S. Molecular Cloning, Purification and Functional Implications of Recombinant GST Tagged hGMCSF Cytokine. Appl. Biochem. Biotechnol. 2013, 169, 1713–1726.
(43) Yao, Q.; Luo, Z.; Yuan, X.; Yu, Y.; Zhang, C.; Xie, J.; Lee, J. Y. Assembly of Nanoions via Electrostatic Interactions: Ion-Like Behavior of Charged Noble Metal Nanoclusters. Sci. Rep. 2014, 4, 3848.
(44) Vanitha, S.; Goswami, U.; Chaubey, N.; Ghosh, S. S.; Sanpui, P. Functional Characterization of Recombinant Human Granulocyte Colony Stimulating Factor (hGMCSF) Immobilized onto Silica Nanoparticles. Biotechnol. Lett. 2016, 38, 243–249.