Electronic Supplementary Information

Protein-Templated Gold Nanoclusters as Specific Bio-Imaging Probe for the Detection of Hg(II) Ions in *In Vivo* and *In Vitro* Systems: Discriminating MDA-MB-231 and MCF10A Cells

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Experimental Details:

Materials

Human Serum Albumin (HSA), gold(III) chloride ($HAuCl_4$), Insulin (human recombinant), sodium hydroxide (NaOH), all L-amino acids and the metal salts were purchased from Sigma Aldrich, USA. MDA-MB-231 and MCF10A cell lines have been purchased from American Type Culture Collection (ATCC). All the culture media were purchased from ThermoFisher Scientific. All the chemicals were of analytical grade and were used without further purification. Milli-Q water was used throughout the experiments for preparing the sample solutions, unless or otherwise mentioned.

Instrumentation

Absorption data were collected using Agilent Cary 100 UV-Vis spectrophotometer. The scanning range was selected from 200 to 800 nm wavelength. Steady-state luminescence measurements were recorded on a Horiba Jobin Yvon Fluorolog 3-111 photometer using a 10 mm path length quartz cuvette keeping the emission and the excitation slits at 2 nm and 1 nm, respectively. For PL lifetime measurements, the AuNCs were excited at 470 nm using N-470L picosecond laser diode (IBH-NanoLED). The full width at half maximum for this particular source was about 160 ps. Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry analyses of the HSA and the NCs were carried out using Bruker Daltonics UltrafleXtreme MALDI-TOF/TOF in the positive ion mode and using Sinapinic acid as the matrix for the measurements. The data was analyzed using FlexAnalysis3.4 software. Transmission Electron Microscopy (TEM) images were obtained from JEOL JEM 2100 F (Field Emission Electron Microscope) operating at 200 kV voltage. Carbon coated Cu grids (300 mesh) was used as substrate for sample preparation. Thermogravimetric Analysis (TGA) was carried out using a Perkin Elmer TGA 4000. 5 mg of the samples were placed in the crucible and subsequently kept inside the furnace compartment. Nitrogen gas was purged (20 $mLmin^{-1}$) during the measurements to maintain an inert atmosphere. The temperature ramp was carried out from 30°C to 800°C at a rate of 10°C min^{-1}. The Fluorescence Correlation Spectroscopy (FCS) experiments were recorded using a Holmarc FCS setup which is attached to an Olympus (IX71) inverted confocal

microscope and equipped with a 60x water-immersion objective having a correction collar (NA 1.20, WD 0.28 mm, model: UPLANSAPO60XW). The excitation of the samples was done using a 532 nm CW DPSS laser (maximum power output: 50 mW) which has a beam diameter <1.5 mm and beam divergence of <1.5 mrad. The fluorescence excitation was passed through a dichroic mirror (Model XF2016, Omega Optical, Inc.) and an emission filter (Model607AF75, Omega Optical, Inc.). The absolute fluorescence signal (exclusively from the sample) was then focused through a tube lens onto a multimode fibre patch cord (Model QMMJ-3S3S-UVVIS-50/125-3-1, Oz Optics) 25 μm in diameter, which acted as the confocal pinhole. The signal was detected by a fibre-coupled single-photon avalanche photodiode (SPCM-AQRH, Perkin Elmer). Autocorrelation of the fluorescence signal was obtained using the hardware attached with FLEX correlator card (FLEX99-0EM). The autocorrelation curves were recorded in a routine written in LabView. The correlation curves were fitted using IGOR-Pro software (WaveMetrics, USA).

Preparation of HSA Templated AuNCs

The protocols for the synthesis of the AuNCs were followed according to our previous reported study.[1,2] Under optimized conditions, the synthesis of HSA protected AuNCs was carried out by adding 5 mL aqueous solution of $HAuCl_4$ (0.01 M) to 5 mL HSA solution (7.5×10^{-4} M) under vigorous stirring. After few minutes, 0.5 mL of NaOH (1 M) was added to the reaction mixture to maintain a pH ~11 which activated the reducing capability of the 18 tyrosine residues in HSA. The mixture was then incubated at 40°C for about 18 hours under vigorous stirring. The formation of AuNCs was indicated by the solution colour change from yellow to deep brown when viewed under ordinary visible light. Then the solution of the NCs was incubated at ~4°C for about 4-6 h and subsequently stored at room temperature for studying their photo-physical and morphological characteristics. It is expected that at low temperature, protein scaffolds will be more encapsulated around the cluster-core which creates more surface stability which in turn makes the NCs more stable (Figure S1).

Ion Sensing and Amino Acid Sensing

The aqueous solutions of the chlorides of Ca^{2+}, Cu^{2+}, Cr^{3+}, Co^{2+}, Cd^{2+}, Fe^{3+}, Hg^{2+}, K^{+}, Mn^{2+}, Mg^{2+}, Na^{+}, Ni^{2+}, Pb^{2+} and Zn^{2+} were prepared. Similar protocol was followed for

preparing all the anionic solutions as well keeping the cation fixed (Na^+ ion). These ionic solutions were then diluted and μL aliquots of those diluted solutions were then titrated with a 2 mL solution of the AuNCs for achieving the desired final concentration. A thorough mixing of the solutions were carried out and then incubated at room temperature (25°C) for about 5 minutes prior to the recording of their PL spectra. The limit of detection (LOD) for the metal ion sensing was estimated from the PL intensity data using the '3σ' rule.[3] The LOD corresponds to the particular concentration of the metal ions where the change in PL intensity of the AuNCs in the presence of metal ions was above three times the standard deviation for the AuNCs in the absence of any metal ions.

Preparation of Insulin-Hg Complex

Aqueous solution of 100 μM Insulin was prepared at basic pH (pH = 11) using 1 M NaOH. In general, Insulin is insoluble at neutral pH and becomes soluble only at high acidic or basic pH. In our study, we adjusted the medium pH to 11 at which Insulin forms a clear solution and simultaneously it also becomes compatible with the pH used for the synthesis of AuNCs. The prepared aqueous solution of Insulin was gradually subjected to addition of Hg(II), and after each addition the mixture was thoroughly shaken, incubated for 1 hour at 25°C, and subsequently monitored by UV-Vis spectroscopy in order to estimate the optimized concentration ratio at which Insulin-Hg complexation occurs.

Fluorescence Correlation Spectroscopic Analysis

For the FCS based ion sensing experiments, the primary step for the analysis involves the dilution of the AuNCs solution to nanomolar concentrations in order to carry out the measurements in the single molecule resolution. After diluting the solution of the NCs, aqueous solution of the Hg(II) were subsequently titrated with this solution such that the starting concentration of the metal ions within the mixture lies in the sub-nanomolar range. After thoroughly shaking the mixture followed by incubation for about 5 minutes, 20 μL droplets of those aliquots were placed on a cover slip which was thoroughly cleaned to remove any impurities, prior to sample addition. The laser power (CW-532 nm) was maintained constantly at an optimum value of ~50 μW to prevent photo-bleaching of the samples. Rhodamine-6G solution in water was used as a standard for the calibration of the

FCS setup; the diffusion time corresponding to the correlation curve obtained for Rhodamine-6G was fitted with equation (1), which gives the value of $\tau_D = 40 \pm 2$ μs. From the reported diffusion coefficient D for Rhodamine-6G in water at 25 °C which is ~4.14×10^{-6} cm^2 sec^{-1},[4] the value of r was calculated as ~258 nm. The fluorescence signal from the samples obtained as autocorrelation functions were fitted by equation (1) to obtain the corresponding values of τ_D.

For a three-dimensional (3-D) Gaussian-shaped observation volume with radial (r) and axial length (l), the normalized auto-correlation function, G(τ) is given by

$$G(\tau)=\frac{1}{N}\left(1+\frac{\tau}{\tau_D}\right)^{-1}\left[1+\left(\frac{r}{l}\right)^2\left(\frac{\tau}{\tau_D}\right)\right]^{-\frac{1}{2}} \quad (1)$$

where N is the average number of particles in the observation volume, and τ_D is the time a particle takes to cross the volume.

The translational diffusion constant (D) can then be related to τ_D as,

$$\tau_D = \frac{r^2}{4D} \quad (2)$$

Cell Culture

MDA-MB-231 (breast cancer cell line) and MCF10A (normal non-cancerous breast derived cell line) were maintained in an incubator at 37°C without and with CO_2 respectively. MDA-MB-231 cells were cultured in L-15 medium supplemented with 10% FBS, 100 μg/mL streptomycin and 100 μg/mL penicillin. MCF10A cells were cultured in DMEM/F12 supplemented with 5% Horse serum, 20 ng/mL EGF, 10 μg/mL Insulin, 100 μg/mL penicillin, 100 ng/mL Cholera toxin, 0.5 mg/mL Hydrocortisone and 100 μg/mL streptomycin. 24 well plate was used to check the cell viability as well as uptake assays. Cells (n = 25000) were grown 24 hour prior to the experiment.

Uptake Assay of AuNCs in Cells and Cell Viability Test

One day prior to experiment, the cultured cells (MDA-MB-231 and MCF10A) were incubated with varying concentrations of the AuNCs (0, 0.5, 1, 10, 30, 50, 70, 100 μM). Cells were observed under the microscope using Trypan Blue staining to check their viability in the presence of the added AuNCs. According to this assay, the live (viable) cells do not incorporate the dye, whereas, the dead (non-viable) cells can take up the staining agent. Thus, the viability of the cells can be estimated from this staining by visualizing the cell morphology.

Cellular Localization

Cells were grown on coverslips in 24 well plate, one day prior to the experiment. MCF10A and MDA-MB231 cells were treated with 30 μM AuNCs for endogenous localization of the clusters. In an independent experiment in MDA-MB-231, AuNCs (30 μM) signal was quenched by treating the cells with Hg(II) (1 μM) for 0, 10, 20, 30 minutes At 25°C, the incubated cells (with Hg(II) and AuNCs) were washed twice with 1X PBS buffer followed by fixation in 4% paraformaldehyde for 15 minutes After fixation, the cells were mounted on glass slides using Mowiol. The images were acquired on Zeiss LSM 780 laser scanning confocal microscope with a 60X objective (oil, 1.42 NA) using 405 nm laser (MBS 405, Emission: 427-478 nm).

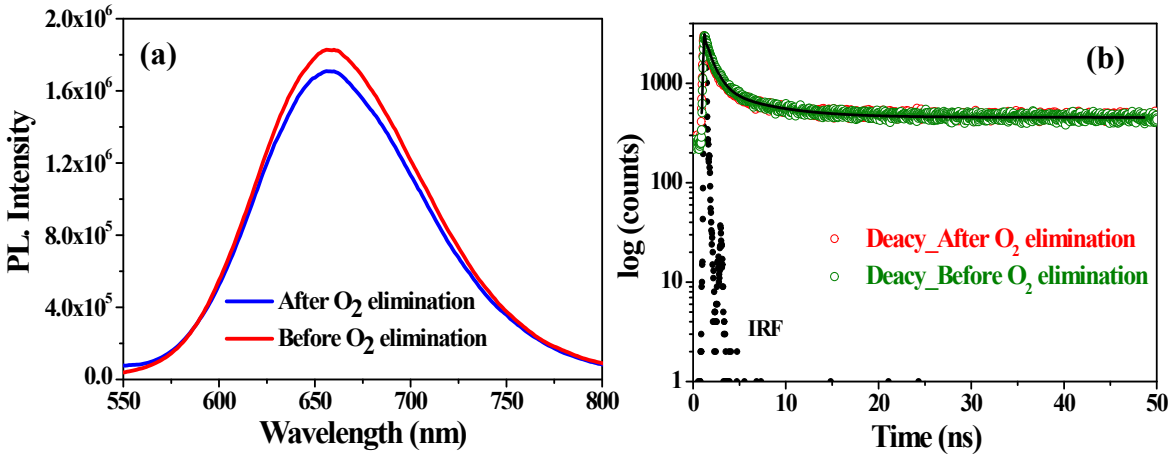

Figure S1. (a) Emission spectra of AuNCs before and after elimination of O_2 as marked in the figure. (b) Excited state lifetime of AuNCs before and after elimination of O_2 as marked in the figure. In both the cases, the oxygen content was removed by the passage of the inert nitrogen gas.

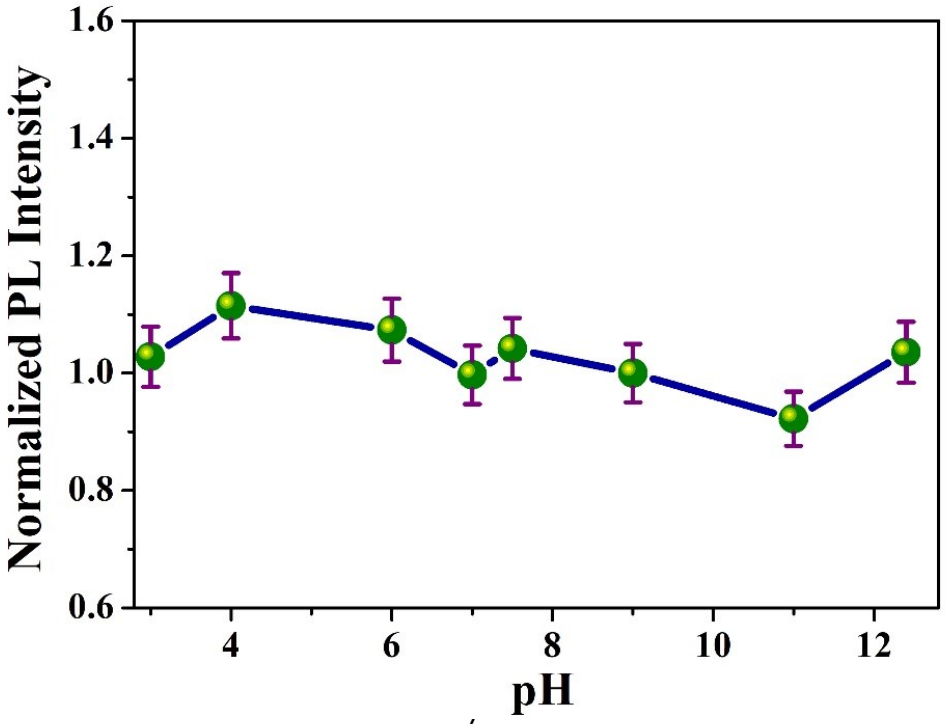

Figure S2. The change of PL intensity of AuNCs with variation of pH of the solution (λ_{ex} = 505 nm, λ_{ex} ~ 660 nm).

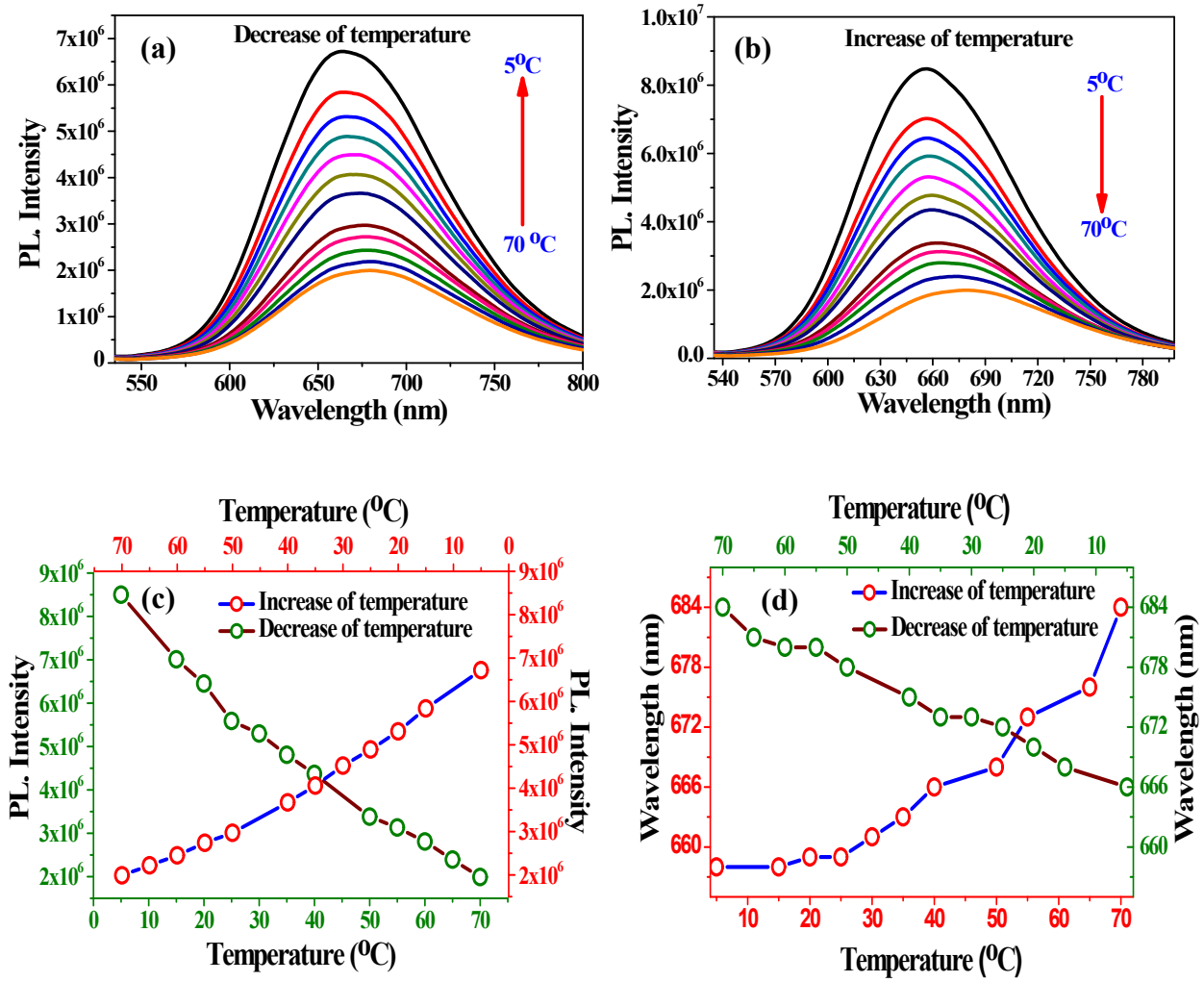

Figure S3. Variation in the emission spectra of AuNCs with (a) decreasing and (b) increasing temperatures. (c) Variation in maximum PL intensity as a function of temperature as marked in the figure. (d) Variation in emission wavelength maxima as a function of temperature as marked in the figure.

Estimation of the number of atoms in a single AuNCs by applying the Jellium model[5]

The theoretical Jellium model was used to estimate the atomic composition of the synthesized metal NCs. The Jellium model states that the number of atoms in a cluster can be predicted using the equation:

$$E_{em} = \frac{E_{Fermi}}{N^{0.33}}$$

where, E_{em}, E_{Fermi} and N correspond to the emission energy of the AuNCs, Fermi energy of the Au and the number of metal atoms, respectively. The values of E_{Fermi} for Au and E_{em} for the AuNCs have been taken as 5.5 eV and 1.88 eV, respectively.

From the above values, the atomic composition of the AuNCs, N was estimated to be ~25.

Formulation of the calibration curve for the quantitative detection ability of Hg(II) by the HSA templated AuNCs:[6]

- $I = I_0 - 613.41 \times [Hg^{2+}]$ for quenching of Photoluminescence up to 26%; ($R^2 = 0.92$)
- $I = 0.26 \times I_0 - 1.45 \times [Hg^{2+}]$ for quenching of Photoluminescence > 26%; ($R^2 = 0.96$)

where I_0 and I are the photoluminescence (PL) intensities of the AuNCs in the absence and presence of the Hg(II). An error of ±5% is associated with these curves based on the accuracy of reproducible results after repetitive experiments. R^2 represents the regression coefficient for the linear calibration curves.

Table S1. Time-resolved PL lifetime parameters of the AuNCs in the presence of increasing concentrations of Hg(II) ions.

Concentrations of Hg(II) added to the AuNCs (nM)	**a_1**	**a_2**	**a_3**	**τ_1 (ns)**	**τ_2 (ns)**	**τ_3 (ns)**	**$<\tau>^{\#}$ (ns)**	**χ^2**
0	98.24	1.30	0.46	346.10	3.01	0.36	340.05	1.02
10	98.00	1.52	0.48	310.70	2.85	0.37	304.53	1.01
50	97.89	1.55	0.56	286.60	2.97	0.36	280.60	1.03
100	97.20	2.06	0.74	264.50	2.99	0.37	257.16	1.05
500	89.96	7.28	2.76	201.30	3.08	0.38	181.32	1.06
1000	86.80	9.56	3.64	167.90	3.07	0.38	146.04	1.06
10000	91.81	5.80	2.38	138.80	3.03	0.51	127.62	1.07
100000	35.83	46.14	18.04	7.06	1.85	0.25	70.43	1.10
500000	47.85	36.58	15.57	4.74	1.39	0.32	2.83	1.11
1000000	49.33	33.90	16.77	4.57	1.21	0.25	2.70	1.08

χ^2 denote the goodness of the fit

$^{\#}$ ±5%

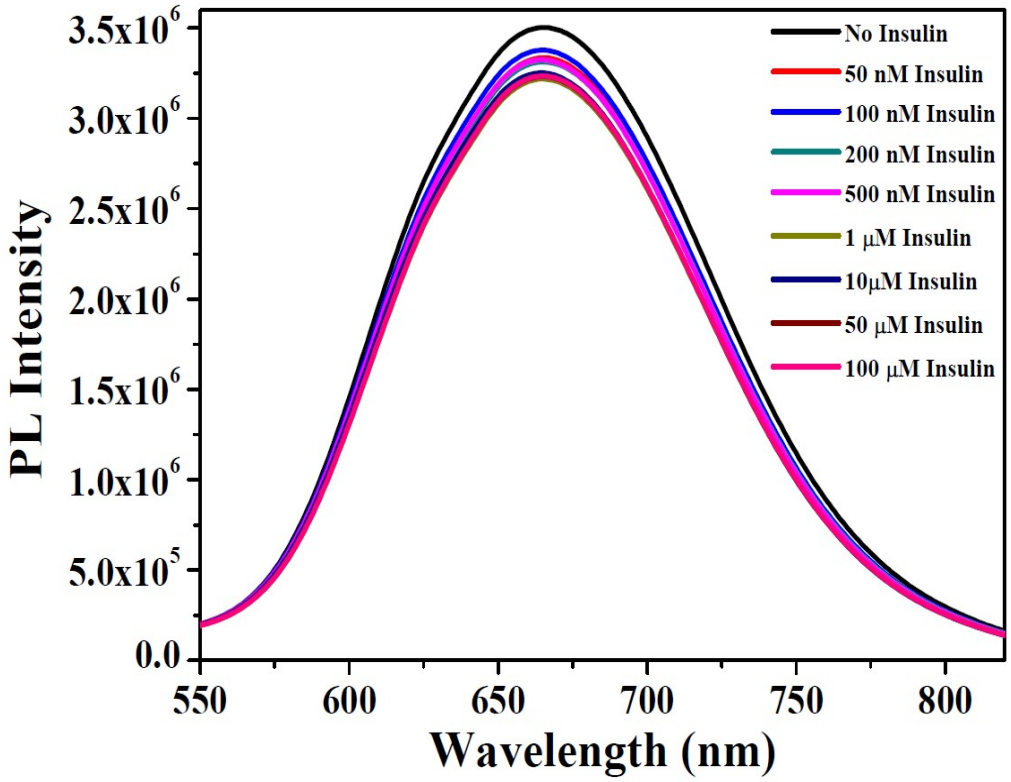

Figure S4. Emission spectra of the AuNCs in the presence of increasing concentrations of Insulin solution as marked in the figure.

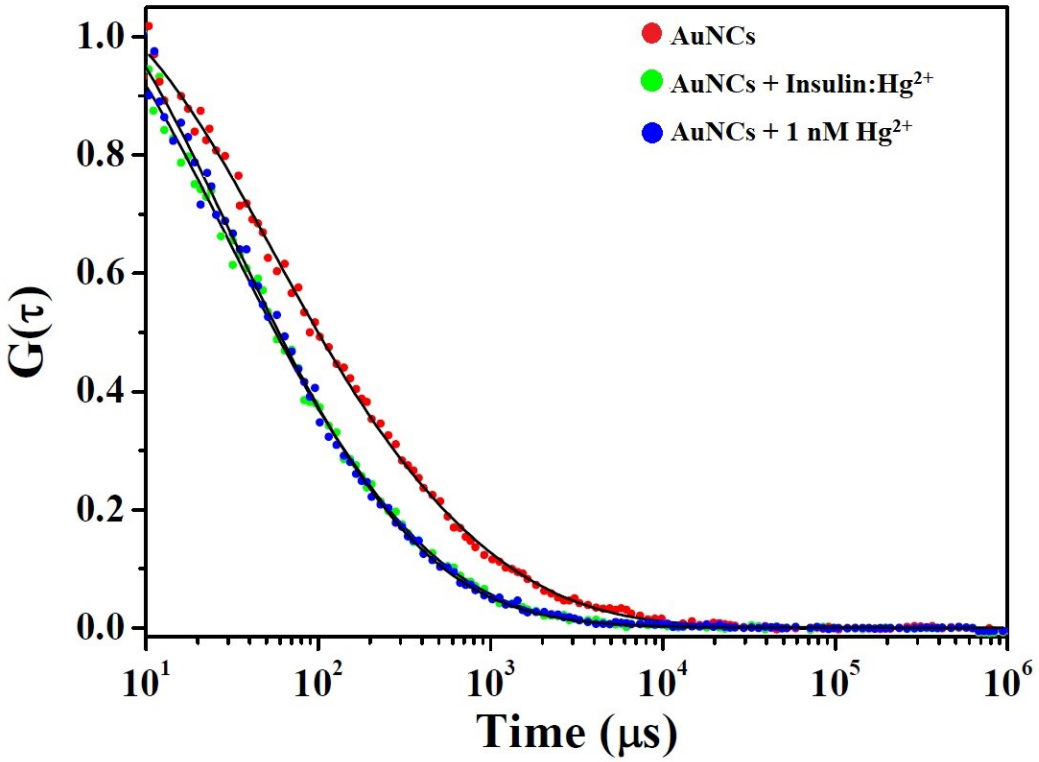

Figure S5. Fitted auto correlation curves for AuNCs alone (red), in the presence of 1 nM Hg(II) (green), and in the presence of Insulin-Hg complex (blue) as marked in the figure.

Table S2. Variation in the diffusion time of the AuNCs in the presence of increasing concentrations of Hg(II), after a period of 1 year and in the presence of 1:1 molar solution of insulin-Hg complex.

Concentrations of Hg(II) added to the AuNCs (nM)	**Diffusion time, τ_D [$]** **(μs)**	**% Decrement in τ_D**
0	798	
0.01	770	
0.05	711	
0.1	678	
1	656	18
10	533	
100	351	
500	273	
1000	-	
AuNCs after 1 year	810	
Presence of 1:1 insulin-Hg complex	679	15

[$] ±5%

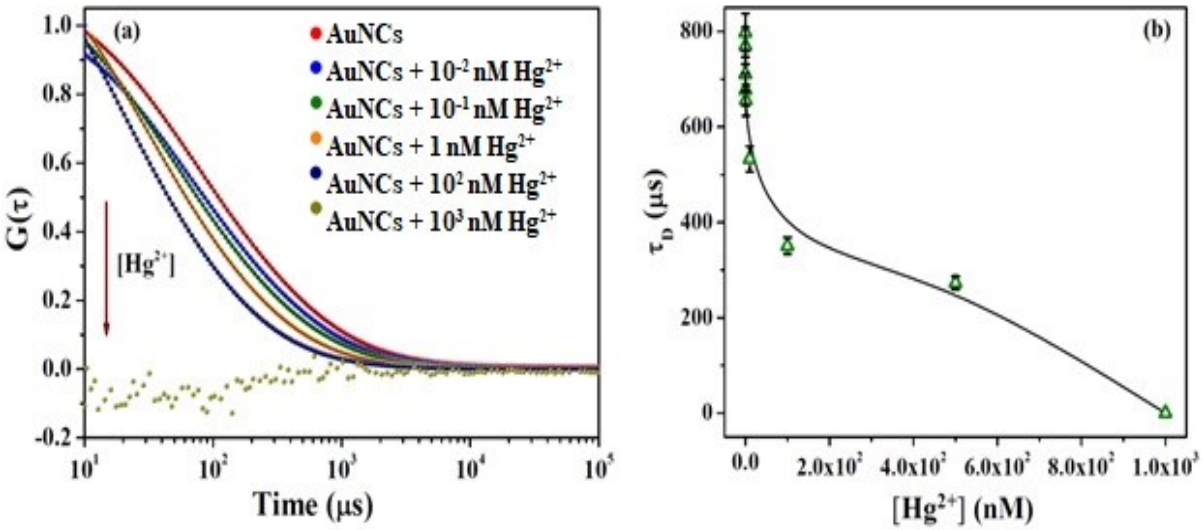

Figure S6. (a) Fitted auto correlation curves for AuNCs with increasing concentrations of Hg(II) as marked in the figure. (b) The variation in translational diffusion times of AuNCs as a function of Hg(II) concentration.

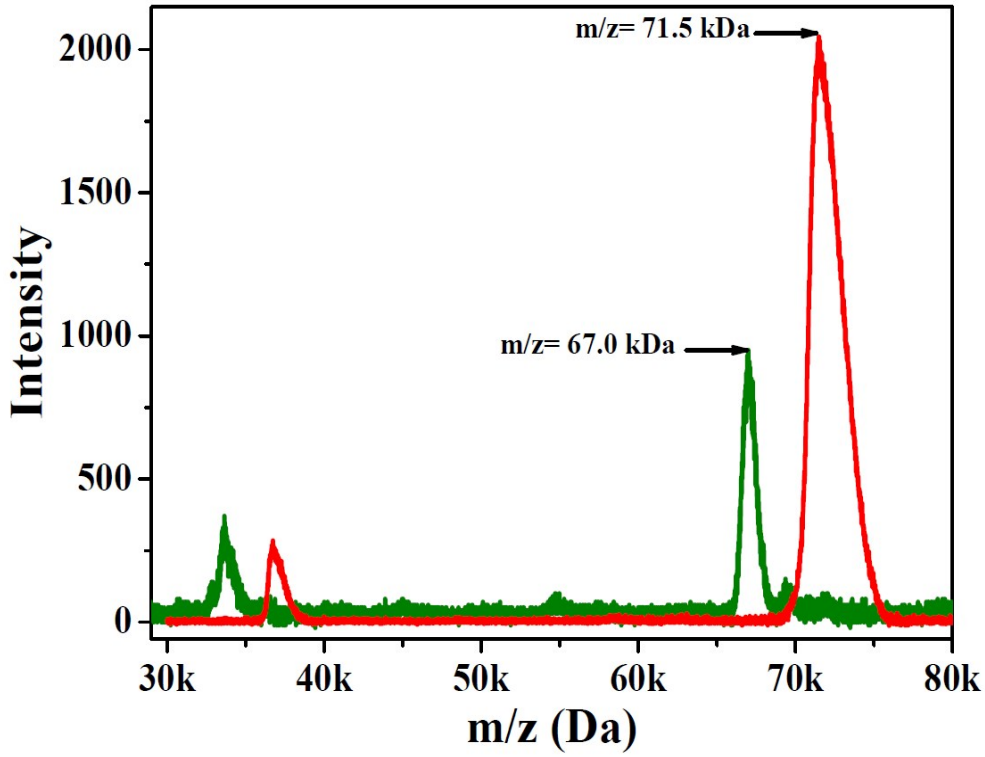

Figure S7. MALDI-TOF spectra of AuNCs in the absence (red spectra) and presence (green spectra) of Hg(II) as marked in the figure.

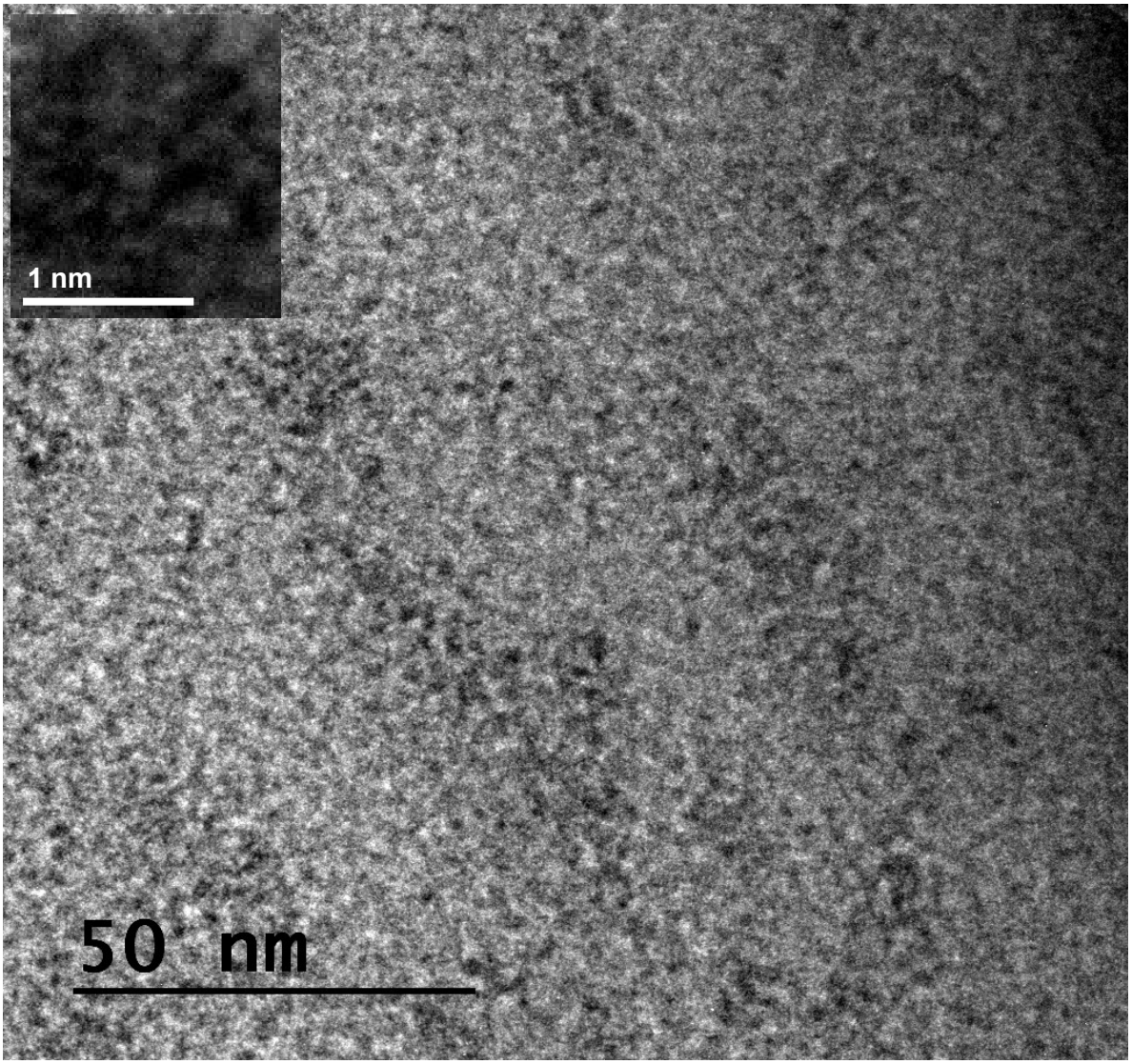

Figure S8. TEM image of the AuNCs after the addition of Hg(II). The inset show high resolution TEM image of a single AuNC after the addition of Hg(II). The size of the clusters was found to be between 1 nm - 1.5 nm.

For estimating the Limit of Detection (LOD) for the Hg(II) using Fluorescence Correlation Spectroscopy:

The LOD was calculated using the following equation.[3,7]

$$LOD = 3\sigma/k \quad (1)$$

The Standard Deviation (σ) is given by,[8]

$$\sigma = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}\left(x_i - \mu\right)^2} \quad (2)$$

where, σ is the standard deviation of blank measurement (i.e., in the absence of any added metal ions), μ is the mean of the parameters, N is the number of parameters, x_i are the magnitudes of the parameters and k is the slope of the calibration curve obtained from linear dynamic plot of fluorescence intensity vs. $[M^{n+}]$.

The measurements of translational diffusion times as tabulated below were done 5 times in the absence of Hg(II). This serves as the "blank measurements".

No. of measurements	**τ_D (μs)**
1	798
2	801
3	795
4	804
5	798

Thus, using equation (2), the Standard Deviation (σ) of blank measurements = 3.42 μs.

The slope of the calibration curve (the parameter k of equation (1)), was obtained 1166.13 μs /nM.

Therefore, LOD = 3σ/k

= 3 × 3.42 / 1166.13 nM

= 0.009 nM

≈ 0.01 nM.

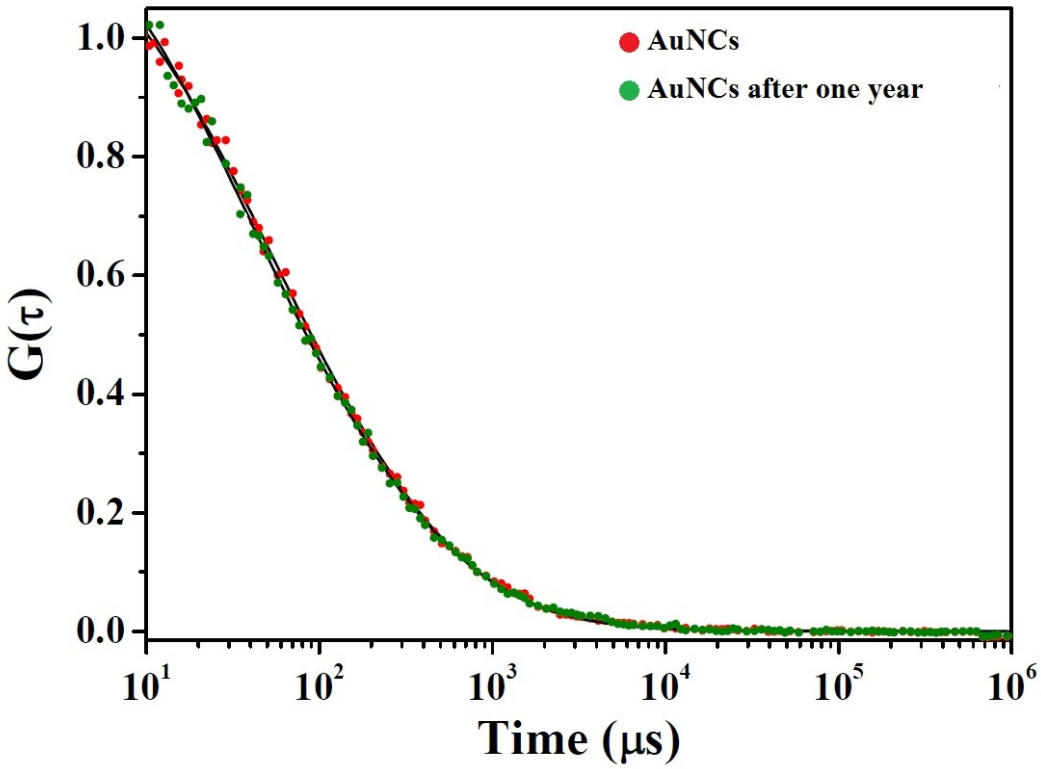

Figure S9. Fitted auto correlation curves for AuNCs after synthesis (red) and after one year (green) as marked in the figure.

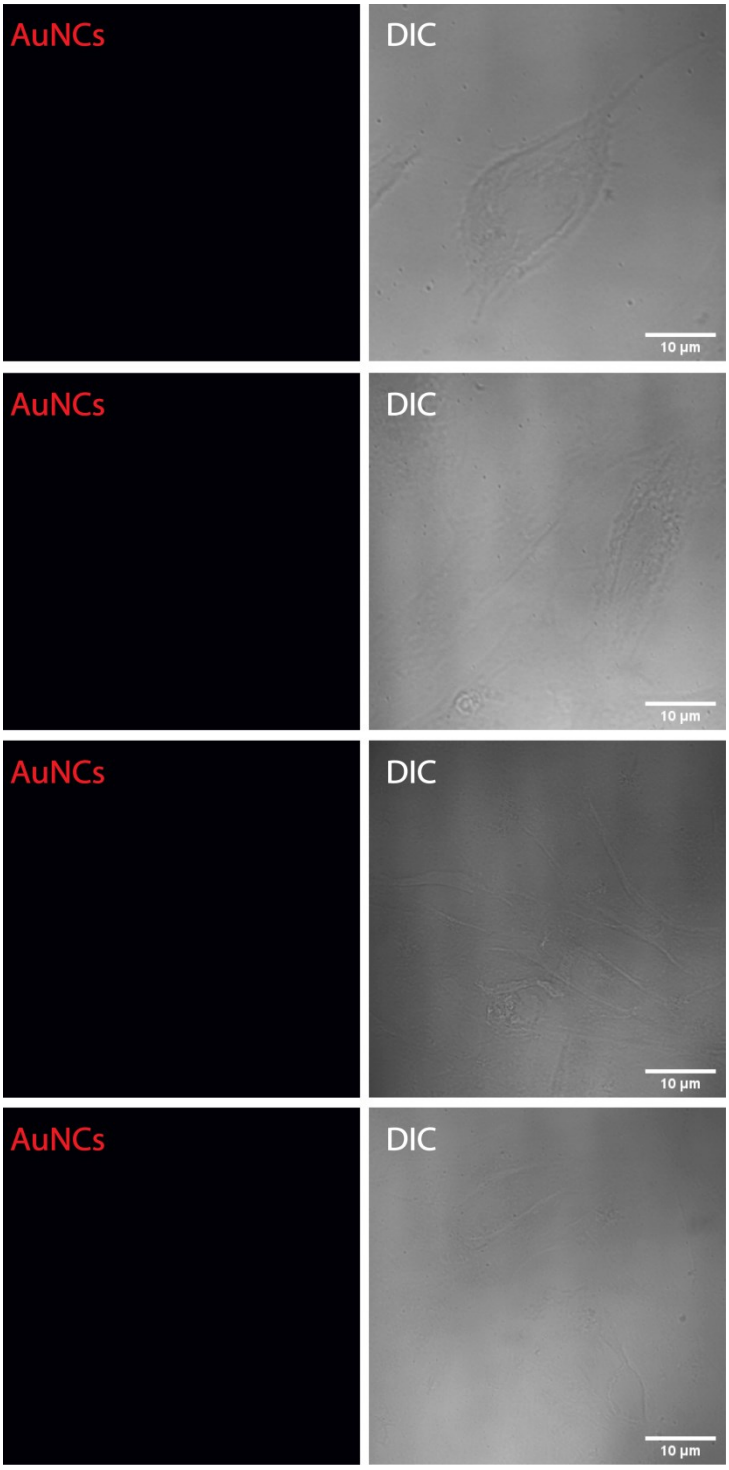

Figure S10. Gallery of confocal images of MCF10A cell line treated with AuNCs.

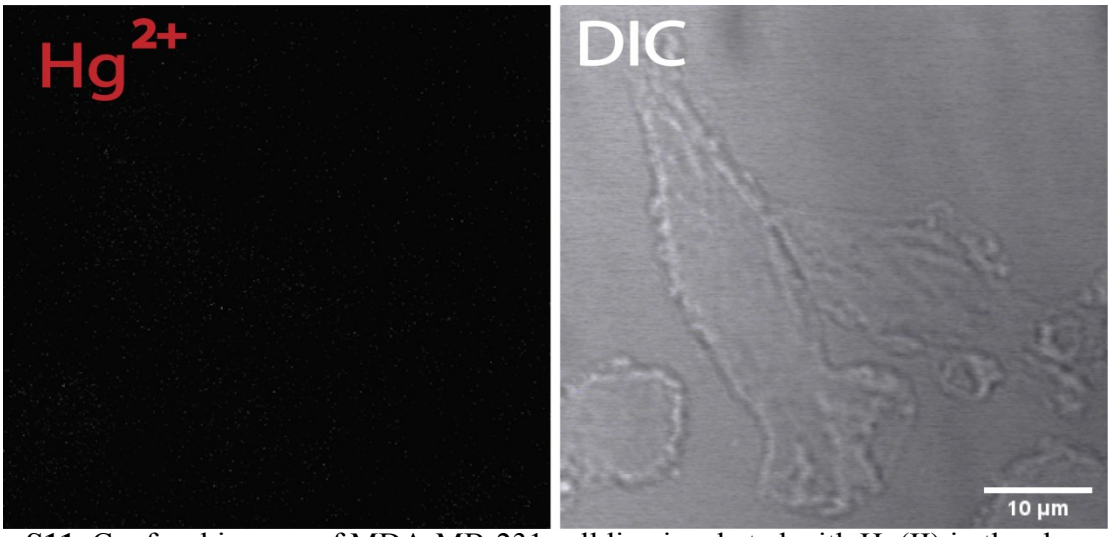

Figure S11. Confocal images of MDA-MB-231 cell line incubated with Hg(II) in the absence of AuNCs.

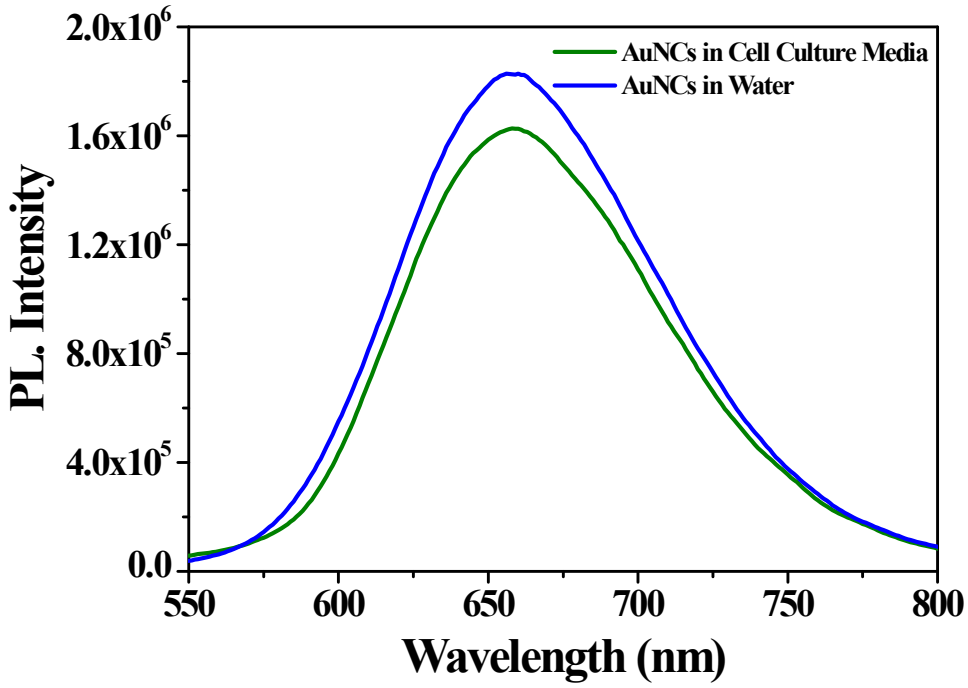

Figure S12. Emission spectra of AuNCs in water and Cell culture medium as marked in the figure.

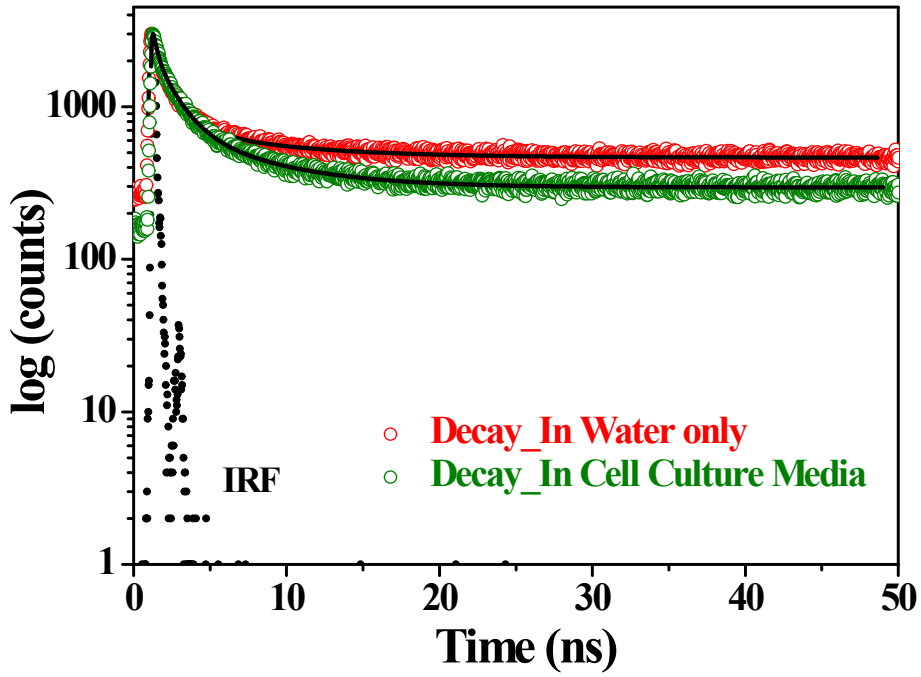

Figure S13. Excited state lifetime of AuNCs in water and Cell culture medium as marked in the figure. In both the cases, the AuNCs are half diluted to maintain the similar concentration in both the solution.

References

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