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ACS Appl. Mater. Interfaces, **Just Accepted Manuscript** • DOI: 10.1021/acsami.7b18837 • Publication Date (Web): 16 Jan 2018

Downloaded from <http://pubs.acs.org> on January 17, 2018

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DNA Damage and Apoptosis Induction in Cancer Cells by Chemically Engineered Thiolated Riboflavin Gold Nanoassembly

Abhishek Sau[†], Sulagna Sanyal^{†,§}, Kallol Bera^{†,§}, Sabyasachi Sen^{†,§}, Amrit Krishna Mitra^{†,†,§}, Uttam Pal^{†,§}, Prabal Kumar Chakraborty[#], Sayantan Ganguly[‡], Biswarup Satpati[±], Chandrima Das^{‡,*}, Samita Basu^{†,*}

[†]Chemical Sciences Division, [‡]Biophysics and Structural Genomics Division, [#]Crystallography and Molecular Biology Division, [±]Surface Physics and Material Science Division, Saha Institute of Nuclear Physics, Kolkata, India, 700064, HBNI, [‡]Department of Chemistry, Singur Government College, Hooghly, West Bengal, India, 712409.

KEYWORDS: Riboflavin Gold Nanoassembly • Nuclear Targeting • DNA Damage Response • Apoptotic Cell Death • Global Gene Expression

ABSTRACT: Herein we have engineered a smart nuclear targeting thiol-modified riboflavin-gold nano assembly, **RfS@AuNPs**, which accumulates selectively in nucleus without any nuclear-targeting peptides (NLS/RGD) and shows photophysically *in vitro* DNA intercalation. A theoretical model using Molecular Dynamics has been developed to probe the mechanism of formation and stability as well as dynamics of the **RfS@AuNPs** in aqueous solution and within DNA microenvironment. The **RfS@AuNPs** facilitate the binucleated cell formation that is reflected in the significant increase of DNA damage marker, γ -H2AX as well as the arrest of most of the HeLa cells at pre-G1 phase indicating cell death. Moreover, a significant upregulation of apoptotic markers confirms that the cell death occurs through apoptotic pathway. Analyses of the microarray gene expression of **RfS@AuNPs** treated HeLa cells show significant alterations in vital biological processes necessary for cell survival. Taken together, our study reports a unique nuclear targeting mechanism through targeting the riboflavin receptors, which are upregulated in cancer cells and induce apoptosis in the targeted cells.

Introduction

The therapeutic applications of nanoparticles in regulating the live cell functions have become the most challenging aspects of nano-biotechnology^{1,2} and nanomedicine^{3,4}. The gold nanoparticles (**AuNPs**) have received considerable attention in the biological and biomedical communities due to their potential use in diagnostic and therapeutic applications^{3,5-8}. The **AuNPs** are usually conjugated with drugs to enhance the efficiency of targeted delivery, thus hold great promises in tumour therapy and drug delivery due to their ability to serve as biocompatible scaffolds for intracellular targeting^{9,10}. Moreover, their nanoscale size allows them to enter cells and to target the different organelles i.e. endosome, nucleus and mitochondria¹¹⁻¹³. The riboflavin (**Rf**) is widely distributed in human tissues in free or conjugated forms and plays an important role in formation of flavoprotein enzyme^{14,15}. The **Rf** that consists of an isoalloxazin moiety and a ribityl chain, possesses mixed features of hydrophobic and hydrophilic functionalities^{16,17}, which generate its affinity towards different biological macromolecules including proteins and DNA¹⁸. Moreover, the **Rf** receptor protein constitutes one type of tumour biomarkers due to their over expression in malignant cells of human breast and prostate cancers^{19,20}.

Scheme 1: Synthetic route of **RfS@AuNPs**

Taking advantage of the above properties of **Rf** and **AuNPs**, we have attempted to design and synthesize one of their modified conjugates, **RfS@AuNPs** which could facilitate significant photophysical changes due to DNA intercalation *in vitro*. Their smart nuclear targeted accumulation in HeLa cells can perturb the highly regulated cell cycles leading to

Figure 1 (A) TEM micrograph of **RfS@AuNPs**. (B) Absorption spectra of **RfSH** (5×10^{-6} M), **CAuNPs** (0.25 nM) and **RfS@AuNPs** (before and after removing free **RfSH**). Inset shows the change in fluorescence intensity of **RfSH** up on addition of **CAuNPs** and subsequent removal of free **RfSH** (C) IR spectra of **RfSH** and **RfS@AuNP**. Inset diagram shows the disappearance of S-H band after nano conjugation.

DNA damage and ultimate cell death. We have also attempted to elucidate the pathway of cell death by global gene expression through microarray analysis.

Results and Discussion

Optical properties of **RfS@AuNPs**:

The terminal hydroxyl group of ribityl chain of **Rf** has been modified due to lack of suitable functional group required for the covalent attachment with citrate capped AuNPs (**CAuNPs**) without affecting isalloxazine moiety. This modification is carried out by dimercaptoethane through tosylation (1) followed by substitution (2) which converts **Rf** to **RfSH** (Scheme 1). Now **RfSH** can easily be attached on the surface of 20-25 nm **CAuNPs** by ligand exchange through Au-S bond resulting in the formation of **RfS@AuNPs** (Scheme 1, Figure 1A, S1).

The absorption spectra of **Rf** and **RfSH** show two maxima at 368 nm and 444 nm (Figure S2A) which remain more or less unaffected with increasing [**CAuNPs**] (0.25-1.5 nM) in 5 μ M aqueous solution of **RfSH** (Figure 1B, S2B); hence, no significant interaction between isalloxazine moiety and **CAuNPs** (TEM Figure S4B) might be inferred. Free **RfSH** is removed from the complex by centrifugation followed by washing and the resuspended solution is used for further experiments after sonication (Figure 1B). The red shift of the **CAuNPs** Plasmon peak by ~ 4 nm at higher concentration of nanoparticles is observed probably due to change in the dielectric environment via surface attachment of **RfSH** (Figure S2B). It has been estimated from the absorption spectra that ~ 22800 **RfSH** molecules are attached with each **AuNP** which is much higher than that for **Rf** [~ 4680] (S4/Figure S3). It is now evident that **RfSH** has ~ 5 times greater binding affinity towards **CAuNP** compared to **Rf** (section S4). Again the fluorescence maximum of **Rf** ($\lambda_{\max} = 524$ nm) is very close to the absorption maximum of **CAuNP** ($\lambda_{\max} = 525$ nm). Therefore energy transfer may be responsible for quenching of

fluorescence of **Rf** with gradual increase of [**CAuNPs**] (section S6, Figure S5). The fluorescence lifetimes (τ) of **Rf** and **RfSH** in aqueous medium are 4.73 ns and 4.69 ns respectively (Figure S6) which remain more or less unchanged with the addition of higher [**CAuNPs**] (1 nM). This suggests that the fluorescence quenching of **Rf** or **RfSH** is a static one due to the formation of ground state complex with **AuNPs**. The attachment of **Rf** and **RfSH** on **AuNPs** surface has been verified further by FT-IR (Figure 1C) where the peak of **RfSH** at 2552 cm^{-1} disappears after the formation of **RfS@AuNP**. The above results indicate that the complex formed between **RfSH** and **AuNP** in ground state is quite stable and might be able to interact with the biological macro-molecules like DNA.

In vitro DNA intercalation by **RfS@AuNPs**

The isalloxazine moiety of **Rf** is able to intercalate DNA due to planer structure^{21,22}. Therefore we have studied binding interaction of **RfS@AuNPs** with calf thymus DNA (ct-DNA) as a model system. With increasing [**RfS@AuNPs**] the absorption maximum of ct-DNA at 257 nm is blue shifted by ~ 15 nm which is higher w.r.t. **Rf** (~ 4 nm) as well as **Rf@AuNPs** (~ 8 nm) (Figure 2A, S7). Upon excitation ($\lambda_{\text{ex}} = 444$ nm) a regular increase of fluorescence intensity with the gradual addition of ct-DNA (10-50 μ M) is observed for a fixed [**RfS@AuNPs**] (Figure S8). This fluorescence enhancement occurs due to stacking interaction of isalloxazine moiety with the base pairs of DNA, which forces them to enter inside the more constrained environment that reduces nonradiative decay. The binding constant of **RfS@AuNPs** with DNA ($K_a = 1.1 \times 10^4 \text{ M}^{-1}$) is ~ 4 times greater than that with bare **RfSH** ($K_a = 2.8 \times 10^3 \text{ M}^{-1}$). This further supports better intercalation of **RfSH** inside DNA as a nano-conjugate (Figure S8C).

Figure 2 (A) Absorption spectra of 10 μM DNA titrated with increasing concentration of **RfS@AuNPs** in tris-buffer (pH 7.2, 10mM). (B) CD spectra of ct-DNA titrated with increasing [**RfS@AuNPs**] (0.1-0.7 nM) in tris-buffer (C) Time-resolved transient absorption spectra of **RfS@AuNPs** (1 nM) (black) in presence of increasing [ct-DNA] in tris-buffer. (Inset: comparative decay profiles of **RfS@AuNPs** (1 nM) without and with ct-DNA (40 μM) with decrease in average lifetime τ from 2.8 $\rightarrow$ 2.1 μs at 360 nm and from 2.9 $\rightarrow$ 2 μs at 530 nm; (λ_{ex} = 355 nm).

The CD spectra of ct-DNA show its conformational changes upon addition of **Rf**, **RfSH**, **Rf@AuNPs** and **RfS@AuNPs** with significant alterations in the positive CD bands in tris-buffer (pH = 7.2). The intensities of the characteristic bands of ct-DNA at 245 nm and 277 nm show changes with the appearance of a new positive CD band around 340 nm (Figure 2B). The intensity of negative CD band increases gradually as a consequence of excess of **Rf**, **RfSH**, **Rf@AuNPs** and **RfS@AuNPs** (Figure S9) in the solution suggesting the probability of occurrence of unwinding of the DNA helix²³.

Moreover, the new band around 340 nm might be induced from **RfS@AuNPs** bound to DNA helix. Recently, it has been reported that a similar positive CD band is observed beyond 310 nm for dinuclear complexes due to selective binding of **RfS@AuNPs** to guanine residues of ct-DNA²⁴. Therefore, it is worthy to mention here that these **Rf**, **RfSH**, **Rf@AuNPs** and **RfS@AuNPs** bind ct-DNA in the ground state. However, it is observed that **RfS@AuNPs** are much more potent intercalator among the others.

The laser flash photolysis experiments have also been performed to study the excited state interaction between ct-DNA and **RfS@AuNP**. The triplet-triplet transient absorption spectrum of **RfS@AuNPs** shows peaks around 360 nm and 530 nm in tris-buffer (pH = 7.2) (Supplementary section S11; Figure 2C). It is observed that with [ct-DNA] (10, 30, 40 μM) the overall triplet absorbance of **RfS@AuNP** drops off along with a comparable decrease in the lifetime (τ) of the transients at 360 nm and 530 nm from 2.8 $\rightarrow$ 2.1 μs and 2.9 $\rightarrow$ 2 μs respectively. The reduction of absorbance and lifetime of the transients may be due to (1) the inaccessibility of the chromophore i.e., isoalloxazine which is stacked within DNA micro- environment²⁵ and (2) the formation of ground state complex between **RfS@AuNPs** and DNA which perturbs the overall inter system cross over processes. Hence triplet yield of **RfS@AuNPs** decreases. The transient state data also reflect the ground state interaction via incorporation of **RfS@AuNPs** within DNA microenvironment.

We have also performed stability assays of **RfS@AuNPs** at different pH (5.0, 7.2 and 8.5) employing fluorescence

spectroscopy (Figure S12) which indicates that the nanoconjugate is quite stable at pH 5.0, 7.2 and 8.5 after 18 hrs which may also be true for **RfS@AuNPs** in lysosomal environment²⁶ where pH is around 5.0.

Simulation of DNA- **RfS@AuNP** intercalation

To probe the stability as well as dynamics of the **RfS@AuNPs** in aqueous solution and within DNA microenvironment, we have built theoretical model using Molecular Dynamics (MD). The structure of **RfS@AuNPs** has been modeled and shown in Figure 3A. A small gold cluster such as Au_{144} cluster is selected as model of **AuNPs** which can bind to a maximum of 60 **RfSH**. A larger particle of about 20 nm diameter is able to accommodate extremely large number of **RfSH** on it. Dynamics of **RfSH** on the gold nano surface has been probed and is shown in the video S1. The ring structures of **RfSH** form stacking interactions with each other to get stabilized on the **AuNP** surface in presence of water. **Rf** is known to intercalate DNA followed by DNA strand break via generation of superoxide²⁷. Therefore, in conjugation to **AuNP**, when **Rf** comes in contact with DNA, the planer ring of **Rf** intercalates within the bases of DNA. Thus, a single molecule of **RfS@AuNP** via intercalation through its multiple head groups can wrap multiple strands of DNA around it. Here, Figure 3B shows the docked structure of **RfSH** into the DNA. MD simulation on this complex reveals that the stacking is energetically stable over time and provides a strong binding force (Video S2). If the tail of **RfSH** is immobilized on a surface like that of **AuNP**, it can immobilize DNA on that surface. An atomic model of that complex has been developed and the geometry is optimized. Figure 3C shows the molecular model of $\text{Au}_{144}(\text{RfS})_{16}$ recruiting two strands of DNA via intercalation. The **RfS@AuNP** bound to two strands of DNA was subjected to MD simulation for 12 ns in aqueous media and found to be energetically stable for the whole duration as shown in video S3.

Figure 3 (A) Molecular model of $\text{Au}_{144}(\text{RfS})_{16}$ or model **RfS@AuNPs** (B) docked structure of **RfSH** into the DNA. (C) Molecular model of $\text{Au}_{144}(\text{RfS})_{16}$ recruiting two strands of DNA via intercalation.

Effect of RfS@AuNPs on live cells

We hypothesized that **CAuNP** functionalized with **RfSH**, may influence their distribution pattern in HeLa cells through the interactions of **Rf** with appropriate receptors inside the cells^{28,29}. Further **Rf** was shown previously to induce apoptosis^{30,31}. The average size of **RfS@AuNPs** (20nm Figure 1A) is lesser than the nuclear pore size (>90nm)^{32,33}. Moreover these molecules are quite stable in physiological condition as shown from MD-simulation, hence can easily penetrate into the nuclei of the cells. To prove the above hypothesis, HeLa cells have been treated with **Rf**, **RfSH**, **Rf@AuNPs** and **RfS@AuNPs** and their cellular uptakes have been monitored by their auto fluorescence ($\lambda_{\text{em}} = 525\text{nm}$) in a time course dependent manner (Figure 4A-H). After 18 H, the relative fluorescence intensity analyses show the trend for nuclear uptake as **RfS@AuNPs** > **Rf@AuNPs** > **RfSH** $\cong$ **Rf** (Figure 4I). The nuclear uptake of **RfS@AuNPs** to the cells is markedly improved upon **AuNP** conjugation [$\sim 20\%$ > **Rf**]. **RfS@AuNPs** predominantly illustrates nuclear localization between 18-24 hrs time points (Figure 4A-D). The cells become much more rounded off in shape 12 H onwards. Post 18 H time point depicts cellular membrane blebbing^{34,35} and formation of the binucleated apoptotic bodies^{36,37} (Figures 4 M-Q) which are the signatures of apoptosis by **RfS@AuNPs**. The live cell imaging provides us with a more in-depth view of the process (Video S4). It is to be mentioned that the unconjugated **RfSH**, **Rf** and **CAuNP** do not show any significant change even after 24 H treatment (Figures 4 J, K, L).

We have quantified the nuclear and cytosolic distribution of **Rf@AuNPs** and **RfS@AuNPs** at each time point of the confocal images (Figure S11). We observe a preferential nuclear localization of **RfS@AuNPs** which peaks at 24 H time point, unlike **Rf@AuNPs**. We have further checked the induction of specific epigenetic signature in the context of DNA damage response upon treatment with **Rf@AuNPs** and **RfS@AuNPs**. Histone H2AX is a variant of H2A histone that is phosphorylated to form $\gamma\text{-H2AX}$ in response to double strand DNA breaks^{38,39}. Figures 5A-F show confocal images of HeLa cells in which the nuclei are stained with DAPI (blue) and anti- $\gamma\text{-H2AX}$ antibodies are with red. For cells treated with 0.5 nM **RfS@AuNPs**, bright-red DNA damage foci

(Supplementary section S2e, Figure 5B) are observed in the nuclei in comparison to untreated cells (Figures 5D-F).

Figure 4: Real time confocal images of 0.5 nM **RfS@AuNPs** treated HeLa cell captured at (A) 6 H (B) 12 H (C) 18 H (D) 24 H (100X objective; Scale Bar represents 20 μm) and (E) 6 H (F) 12 H (G) 18 H (H) 24 H for **Rf@AuNPs** treated HeLa cells (Scale Bar represents 20 μm) (I) Relative green fluorescence Intensity of **RfSH**, **Rf@AuNPs**, **RfS@AuNPs**, **Rf** fluorescence in the HeLa nucleus after 12H treatment. (J, K, L) Real-time images of cancer cells (HeLa) at maximum time point (24 H) after treatment of (J) **CAuNP** (K) Only **Rf** (L) only **RfSH** (Scale Bar represents 20 μm); Panel: M, N, O, P, Q are the representative time-lapse images of a HeLa cell treated with 0.5 nM **RfS@AuNPs** undergoing membrane blebbing and binucleated (indicated by arrow) cell formation. (The scale bar is 10 μm .)

These results have been reconfirmed by immunoblotting experiments (Figure 5I). The DNA double strand break can be caused by various cellular events like replication fork collision, apoptosis, external damage and dysfunctional telomeres that may lead to phosphorylation of H2AX^{40,41}. Further, the flow-cytometric analysis has been carried out to monitor the ability of **RfS@AuNPs** to modulate cell cycles (Figure 5G, S10B). The Pre-G1 peak shows a huge increase in presence of **RfS@AuNPs** (~ 46.4 % of total cells) treated for 24 H as compared to control untreated cells (~ 4.6 % of total cells). The alteration in the percentage of cells in different stages of cell cycle is represented graphically (Figure 5G, S10). Hence most of the **RfS@AuNPs** treated cells are arrested at pre-G1 phase, indicating cell death^{42,43} more potently as compared to **Rf**. The DNA fragmentation in apoptotic cells are quantified by DNA ladder assay and found to be maximum in case of **RfS@AuNP** treated cells (Figure 5H). The suggested cause for cell death in **CAuNP**-treated cells is the generation of reactive oxygen species (ROS), which is known to cause irreversible DNA damage^{44,45}.

Figure 5. Immunofluorescence staining by γ -H2AX antibody. Confocal image of HeLa cells treated with 0.5 nM **RfS@AuNPs** (A) blue channel (B) red channel (C) merged image of (A) & (B). Untreated HeLa (D) blue channel (E) red channel (F) merged image of (E) & (F). (In 100x-objective, Scale Bar = 20 μ m). (G) Flow-cytometric analysis showed % of cells in each phase (cyan-preG1; blue-G1; red-S; black-G2) of the cell cycle for HeLa cells in the presence of 0.5 nM **Rf@AuNPs** and **RfS@AuNPs**, suggesting cell death. (H) DNA fragmentations in apoptotic cells are quantified by DNA ladder assay (I) Immuno-blotting by γ -H2AX antibody. α -H3 is used as a loading control.

Global gene expression analysis upon **RfS@AuNPs** treatment

Treatment of the **RfS@AuNPs** perturbs a large number of biological processes in the HeLa cells, including vital processes like DNA repair processes, chromatin organization, mitosis, cell cycle etc. It is well evident from the list of affected processes that the gold nano assembly leads to loss of cell viability for the cancer cells. This fact coupled with the selective targeting of the cancer cells through **Rf** receptors renders a unique specificity to the gold nano assembly, and represents a potential mode of modern cancer therapy. Specific targeting and delivery of nano particle can bypass the non specific cytotoxic effect of radiation and chemotherapy, and thus, it is currently a major area of anti cancer drug designing. Our current study sheds light on a unique mode of tumor specific nano particle conjugated drug delivery and also advances our current understanding of nano particle effect on cellular processes. Selective upregulation of pro-apoptotic markers like Bax⁴⁶, IL-6⁴⁷ and subsequent down regulation of anti-apoptotic markers like STAT1⁴⁸ and BCL2L12⁴⁹ (Figure 6 A-H, S13 and S14) also indicate that **RfS@AuNPs** induce apoptosis.

CONCLUSION

Here we have tried to design and synthesize a nanocojugate, **RfS@AuNPs**, which shows four times stronger binding with ct-DNA compared to **Rf**, **RfSH** and **Rf@AuNPs**. The MD-simulation also confirms the stability of the conjugate in aqueous medium. Further, we have shown that the accumulation of **RfS@AuNPs** in the nuclei of the cells may be due to the appropriate over expressed receptor present in the treated HeLa cells. Most of the cells are arrested at pre-G1 phase, inducing DNA damage and ultimately cell death. **RfS@AuNP** has a number of advantages in terms of synthesis and its applications in cells. The **RfS@AuNP** (1) can be easily prepared from the low cost vitamin-B2 just by a simple two step process, (2) has auto green fluorescence which can be easily tracked by simple confocal microscope (i.e. one-photon excitation λ_{ex} = 444 nm), (3) can be used in low concentration as a nucleus targeting agent without attachment of any nuclear localizing signal (NLS/RGD) peptide, dendrimer with **AuNPs** and (4) can deliver drug molecules in the cell nucleus by chemical attachment. **AuNP**-conjugated drug can regulate many cellular processes in a more efficient manner as obtained from analysis of the microarray gene expression of **RfS@AuNPs** treated HeLa cells. **RfS@AuNPs** might find new applications in tumour diagnosis and cancer therapy.

Figure 6. (A) Clustering and heat maps of expression values for differentially expressed genes. Down-regulated genes are marked in green and up-regulated genes are marked in red. From left to right first three samples are control untreated and latter three samples are 0.5 nM RfS@AuNPs treated HeLa cells. (B) Overrepresentation of differentially expressed genes from microarray in cell survival related processes (C) Validation of candidate upregulated and down regulated genes by qRT-PCR after treating the HeLa cells with RfS@AuNPs for 12 H. Normalization was done with 18srRNA and reference level was considered as 1. Each bar is an average of 3 individual biological replicates. Error bars show standard deviations. (D, E) Network of differentially regulated biological processes upon nanoparticle treatment in HeLa cells. Nodes represent individual biological processes, and edges represent the connection between them. Network constructed using BiNGO tool of Cytoscape software. The colour scale represents the level of significance of the GO process (preset cut-off: $p < 0.05$). Overrepresentation of differentially expressed genes from microarray data in following categories: F. Molecular function. G. Biological processes. H. Signaling pathways.

Experimental Section

All the chemicals and solvents required for syntheses of the thiol derivative of Riboflavin are of analytical grade. We have purchased 7,8-Dimethyl-10-[(2S,3S,4R)-2,3,4,5-tetrahydroxy-2-pentyl]benzopteridine-2,4-dione (Riboflavin), 1,2-Ethanedithiol, from Aldrich Chemical Co., highly polymerized calf thymus DNA (ct-DNA) from Sisco Research Laboratory, India and tris buffer, sodium chloride and hydrochloric acid (AR) from Merck. The chemicals have been used without further purification. Triple distilled water has been used for the

preparation of all the aqueous solutions. Solvents required for syntheses and spectroscopic studies have been purchased from SRL, India and Spectrochem, India respectively. HAuCl₄, 3H₂O, trisodiumcitrate has been procured from Sigma. Steady-state absorption and fluorescence spectra of Rf have been recorded using JASCO V-650 absorption spectrophotometer and Spex Fluoromax-3 Spectro-fluorimeter respectively. Time-resolved emission spectra of RfS@AuNPs have been obtained using a picosecond pulsed diode laser based TCSPC fluorescence spectrometer with λ_{exc} ~370 nm and MCP-PMT as a detector. Respective transient intermediates have been generated with third

harmonic (355 nm) output of nanosecond flash photolysis set-up (Applied Photophysics) containing Nd:YAG Laser (Lab series, Model Lab 150, Spectra Physics)⁵⁰. TEM measurements have been carried out using an FEI, TECNAI G2F30, S-TWIN microscope operating at 300 kV equipped with a GATAN Orius SC1000B CCD camera. Flow cytometry has been performed on a FACS Calibur from BD Biosciences.

SYNTHESES

Synthesis of RfSH.

A 500-mL round-bottomed three-necked flask fitted with a stirrer and a thermometer is charged with 200 mL of pyridine and 18.81 g (0.05 mol) of Riboflavin (Rf) in 200 mL of DMSO. The solution is stirred and chilled to 0°C and then 9.53 g (0.05 mol) of freshly recrystallized p-toluenesulfonyl chloride is added in portions over a 20-min period. The reaction mixture is allowed to attain room temperature and is stirred for 16 h. The mixture is re-cooled to 0°C and poured into 100 mL of concentrated hydrochloric acid in 500 mL of ice water. The aqueous layer is extracted with 100 mL of DCM and the combined organic layers are washed with two 60 mL portions of brine, dried over magnesium sulphate and concentrated by rotary evaporation to yield 10.2 g of a light yellow solid of Rf-OTs. Rf-OTs (10.61 g, 0.02 mol) is dissolved in ether (100.0 mL) by continuous stirring and then 1, 2-dithiane (2 g, 0.02 mol) is added in drop wise manner. The reaction mixture is stirred for 24 hours in presence of catalytic amount of NaI (0.003 g, 0.00002 moles) under room temperature. The dark yellow product is purified using column chromatography and used as **RfSH**.

¹H NMR (300 MHz, DMSO-d₆) 11.34 (s, 1H, N12-H), 7.92 (s, 1H, Ar-C1-H), 7.88 (s, 1H, Ar-C4-H), 5.15 (s, N14-H), 4.59 (s, N8-H), 4.93, 4.90, 4.87, 4.8 (m, 1H, 1H, C18, 20-H), 4.80, 4.79 (d, 2H, C17-H), 4.63, 4.59 (d, 2H, C21-H), 3.47, 3.45, 3.43 (t, 1H, C19-H), 2.675, 2.671, 2.66, 2.64 (m, 2H, C24-H), 3.63 (s, 1H, 3-OH), 2.58 (s, 1H, S25-H). 2.57-2.50 (m, 2H, C23-H), 2.48 (s, 3H, C15-H), 2.40 (s, 3H, C16-H). [Figure S1 A]

¹³C NMR (75 MHz, DMSO-d₆): 117.5(C1,C4), 135.7(C2), 136.7 (C3)150.8(C6), 146.1(C7), 132.1 (C9), 134.0(C10), 155.6 (C11), 160.0 (C13), 18.8 (C15,C16), 47.36 (C17), 63.4 (C18), 68.8(C19), 72.8 (C20), 73.6(C21), 40.3(C23), 38.2 (C24). [Figure S1 B]

ESI mass of RfSH: m/z obtained for **RfSH** is: 454.16 (Exact mass 454.13) [Figure S1C]

Preparation of RfS@AuNPs: Aqueous solution of **RfSH** (5×10^{-4} M) is mixed with 1 nM CAuNPs in 1:4 ratio. Resulting solution is kept for ~ 4 h and centrifuged (at 4000 rpm) for 90 minutes. This process is repeated thrice. The bottom part is collected, redispersed in water and used as **RfS@AuNPs** for each experimental step. Concentration used for cell treatment is 0.5 nM.

Methods of MD-simulation: Coordinates of an icosahedral metallic gold cluster comprising 144 Au atoms are obtained from the published work of Lopez-Acevedo et al.⁵¹. DNA intercalated with a thiazole orange dye is obtained from the Protein Data Bank (PDB ID: 108D). Structure of thiooxalated riboflavin (**RfSH**) is drawn in Schrodinger maestro molecular modeling environment. Multiple **RfSH** molecules are attached to the surface of the Au cluster through the Au-S linkage. The **RfSH@AuNP** complex is then subjected to the MD simulation in aqueous phase for 12 ns. Prior to production MD structure is minimized using a previously published five step relaxation protocol⁵². In the DNA structure, 108D, the ligand is exchanged for **RfSH** using Auto Dock 4.2⁵² followed by energy minimization in Schrodinger Maestro. Two DNA strands intercalated with **RfSH** are then attached to the surface of Au₁₄₄ via Au-S linkage. DNA bound **RfS@AuNP**

complex is then solvated in single point charge water model and the dynamics of the system is probed for 12 ns in normal temperature and pressure. Restraints are added to the terminal base pairs to prevent base fraying. All the MD simulations are performed in Desmond (Academic version 2016-4) as implemented in Schrodinger Maestro⁵³ (using previously published protocol)⁵².

Microarray analysis and its validation

Microarray analysis: Raw data are Quantile normalized and baseline transformation is done to median of all samples using Gene Spring GX 12.5 software (Agilent Technologies Inc, Santa Clara, USA).

Statistical analysis and differentially expressed genes: Differentially expressed probe sets (Genes) upon treatment in comparison to untreated cells are identified by applying Volcano Plot using a fold-change threshold of absolute fold-change greater than or equal to 1.5 and a statistically significant t-test P value threshold adjusted for false discovery rate of less than 0.001. Statistically significantly enriched transcripts with a P value adjusted for false discovery rate of less than 0.05 derived using the hyper geometric distribution test corresponding to differentially expressed genes are determined using student t-test with Benjamini Hochberg FDR test. Unsupervised hierarchical clustering of differentially expressed genes upon treatment in comparison to untreated are done using Euclidian algorithm with Centroid linkage rule to identify gene clusters whose expression levels are significantly reproduced across the replicates.

Biological pathways and gene ontology enrichment analysis: Differentially expressed gene list is subjected for biological significance analysis by GOElite tool (Ref). 21887 protein coding genes are used as the background and differentially expressed gene list is used as query. Database of Gene Ontology categories, Wiki pathways, KEGG Pathways, Pathway Commons, Pheno Ontology, Diseases, Protein Domains, Transcription factor targets and Tissue expression are configured for significant analysis. Each query list is subjected to over representation analysis against each of the above databases. A Z score and permutation or Fisher's Exact Test p-value are calculated to assess over-representation of enriched biological categories.

Gene regulatory network modeling: Statistically significant dysregulated genes are used as input for identification of the relevant biological processes. The output file is visualized in Cytoscape V 2.8 in the form of connections along with modeling the network. Genes are subjected to color codes depending on their statistical significance and the major biological processes are highlighted to identify biological pathways. DAVID⁵⁴ and PANTHER⁵⁵ classification tools are also used to ascertain the functional classification of the Differentially Expressed genes.

RNA isolation and qRT PCR: Total cellular RNA is extracted using TRIZOL reagent (Invitrogen) as per manufacturer's instructions and RNA quantity as well as quality is determined using Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The reverse transcription is performed with revertaid first strand cDNA synthesis kit (Thermo Scientific). Primer sequences used in the assay have been provided in the supplementary section. Four genes are chosen for validation of the microarray data: STAT1, BAX, BCL2L12 and IL-6.

ASSOCIATED CONTENT

Supporting Information. It contains details of synthesis and characterisation of **RfSH** and **RfS@AuNPs**. It also contains the Figures S1—S14 and Videos S1—S4. “This material is available free of charge via the Internet at <http://pubs.acs.org>.”

AUTHOR INFORMATION

Corresponding Author

*samita.basu@saha.ac.in

*chandrima.das@saha.ac.in

Present Addresses

KB: Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA 91125

Author Contributions

[§]These authors contributed equally.

ACKNOWLEDGMENT

We sincerely thank S. Haldar, R. Modok, Y. Sikdar, C. Sengupta, P. Mitra, S. Das Chakraborty and M. Bhattacharyya for their constant support and help. We acknowledge financial support from UGC (A. S.; F2-32/1998 (SA-1), DBT- Government of India, Ramalingaswami fellowship and BARD project: DAE at Saha Institute of Nuclear Physics..

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