Supporting Information

Super resolution microscopy revealed the lysosomal expansion during Epigallocatechin Gallate mediated Apoptosis

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Material and Method

Materials

Chemicals and plastic wares procured are EGCG (Sigma Aldrich), Phosphate buffer (Thermo scientific), serum free and serum supplemented DMEM (Gibco), T-25 flask, 96well plate (Thermo scientific), 6 well plate (Themo scientific), and FACS tube (CellPro).

Preparation of Serum albumin stabilize Nano-conjugate

EGCG dissolved in the DMSO to prepare the concentrated solution of EGCG. BSA is dissolved in the 1X PBS buffer. The drug conjugation i.e. BSA-EGCG (1:35 has been prepared upon combining the appropriate ratio of protein and drug molecules and volume adjusted with 1X PBS buffer. The prepared conjugates were kept on a rotator for 50rpm for 3 hr at 20 ºC. Further dialysis of the prepared sample (BSA-EGCG in 1:35 ratio) was performed using the 20kDa dialysis membrane to separate any residual free EGCG.

Calculation of K_d upon EGCG binding to labeled BSA

BSA labeling was performed using the cysteine reactive dye MO-L004 (Contains maleimide group) using a standard protocol of Nano Temper[1]. The dissociation constant (K_d) of the interaction between BSA and EGCG calculated using a Monolith NT.115 device that works on the principle of Microscale Thermophoresis (MST) based fluorescence signal change, which is described as the diffusion of molecules under temperature gradient across capillary result in byre distribution of fluorescent sample upon IR laser activation[2]. The MST signal varies for the bound and unbound state of samples since it is very sensitive for the charge, size, and hydration shell of molecules therefore used for determining the binding affinity[3]. K_d is calculated from the Law of mass action, which is given as follows:

$$K_d = [P]*[L]/[PL]$$

Where [P] is a concentration of protein and [L] the concentration of free ligand and [PL] is a concentration of Complex between P and L. the free concentration of protein and ligand is calculated from given eq. as follows:

$$[P] = [P0]-[PL] \text{ and } [L] = [L0]-[PL]$$

Where [P0] and [L0] is the known concentration of protein and added ligand respectively, to determine the concentration of Bound substrate and drug quadratic functional fitting is applied, the obtained equation is given as,

$$[PL] = 1/2*(([P0]+[L0]+Kd)-(([P0]+[L0]+Kd)^2-4*[P0]*[L0])^{1/2})$$

During the experiment concentration of fluorescently labeled Protein was kept constant and the concentration of ligand varies in sequential dilution series. The signal obtained in the measurement directly corresponding to the fraction of Protein-bound to ligand, which can be fitted with the derived equation to obtained K_d. The K_d value obtained for BSA-EGCG interaction was 2.13µM, this low K_d value represents the high intractability of EGCG to BSA. Change in fluorescence intensity for each ratio of BSA and EGCG automatically fitted by the software provided the K_d value.

Fluorescence Lifetime

The fluorescence lifetime of BSA (200µM) and its conjugation with EGCG in different ratios were performed using the Excitation laser of 284nm and Emission at 345nm. The fluorescence emission decay graph plotted between photon counts and time provided the fluorescence lifetime spectra. Fluorescence lifetime on changing EGCG concentration proves the presence of dynamic quenching. Although the plot of the ratio of initial fluorescence and change fluorescence upon addition of quencher versus quencher i.e., $\frac{F_0}{F} vs\ [Q]$ results in upward curvature and don't exactly fit linearly that indicate **the** presence of Static and dynamic quenching simultaneously and can be fitted in the plot between $\frac{1}{[Q]}\left[\frac{F_0}{F} - 1\right] vs\ [Q]$. As shown in **Figure-S1b**.

$$\frac{F_0}{F} = 1 + K_s[Q] \textbf{ (eq.1)}$$

$$\frac{F_0}{F} = 1 + K_D[Q] \textbf{ (eq.2)}$$

$$\frac{1}{[Q]}\left[\frac{F_0}{F} - 1\right] = [K_S + K_D] + K_S K_D[Q] \textbf{ (eq.3)}$$

Equation 1 and 2 is Stern Volmer Equation for static and dynamic quenching while equation 3 represent when both static and dynamic quenching event occurs simultaneously. On fitting and solving the equation obtained Intercept i.e. $[K_S + K_D]$ is 0.00169 $and\ slope$ $K_S K_D$ is 3.49706 × 10^{-7}. On solving, K_D can have values (roots) = 1.44 x10^{-3} and 2.41 x10^{-4}. we have already obtained the value for K_D 0.0011 from the plot of the ratio of an initial lifetime and change lifetime upon addition of quencher versus quencher i.e., $\frac{t_0}{t}$ vs $[Q]$ which is corresponding to eq.1 fitted in a graph (**Figure-S1c)** and obtained K_D value is near to the 1st root, therefore, obtain quenching constant K_D and K_S is 1.44 x10^{-3} M^{-1} and 2.4 x10^{-4} M^{-1} respectively.

Preparation of Labeled BSA

A solution was prepared of mixing BSA (2mg/ml in final solution) and 10µl of dye (Atto488 NHS Ester (Sigma Aldrich) or Cyanine-3 NHS Ester (Lumiprobe)) in a final volume of 200µl and kept overnight rotating at 50rpm at 18ºC, afterward, the prepared solution was dialyzed using the 20KDa dialysis membrane (Spectrum spectra/por company) for 3 days to purify the Labelled BSA from any unbound (Residual) dye molecules.

Sample preparation for FLIM and SRRF Imaging

HeLa cell ~20,000 in counts, seeded in 6 well plates and after overnight adherence fasted for 6hr by incubating in Serum-free DMEM media. After completion of 6hr cells were treated with BSA, BSA-EGCG Conjugates (In different ratios) in a final concentration of 1µM. dilution of conjugates (or BSA) prepared in DMEM+1%FBS medium. After 10 min. of addition of drug conjugate (or BSA), Lysotracker dye (BSA-Cy3) was added in the final concentration of 1µM for FLIM and 0.1µM for SRRF Imaging. After completion of 24 hr incubation with drug conjugate (or BSA) cells were fixed using 4% Paraformaldehyde and Glycerol-PBS mounting media.

Super-Resolution Radial Fluctuation Microscopy

The SRRF is a threshold-free algorithm based on the analysis of the image sequence. It considers that the image is convolved with a point spread function (PSF). Each of the PSF created by the single molecules contained a higher degree of local geometrical symmetry than the background. It is better than the single-molecule detection because it calculates the local gradient convergence or termed as radiality in the whole frame by dividing each pixel into sub-pixels, which preserve the information in the gradient field which would be discarded by any other localization technique. The radiality distribution is independent of the PSF intensity and the FWHM of this distribution can be adjusted by gradient convergence radius. The full image created by the radiality distribution can acquire the image noise corresponding to nonfluorophore associated radiality peaks, but it can further be de-noised by the time series analysis, the increase in FWHM

of the radiality distribution, or the radiality map waiting with the fluorophore intensity. In the time series analysis, the higher-order temporal cumulants can be calculated and the noise reduction happens because of the uncorrelated noise peaks in the time series and high order correlated peaks at the center of the actual fluorophore[4].

Statistical analysis

Statistical analysis was performed using the GraphPad t-test calculator available online at https://www.graphpad.com/quickcalcs/ttest1.cfm. Analyzed data values from independent experiments presented as mean±SD.

Reference

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(3) Seidel, S. A. I.; Dijkman, P. M.; Lea, W. A.; van den Bogaart, G.; Jerabek-Willemsen, M.; Lazic, A.; Joseph, J. S.; Srinivasan, P.; Baaske, P.; Simeonov, A.; Katritch, I.; Melo, F. A.; Ladbury, J. E.; Schreiber, G.; Watts, A.; Braun, D.; Duhr, S. Microscale Thermophoresis Quantifies Biomolecular Interactions under Previously Challenging Conditions. *Methods*. Academic Press Inc. March 1, 2013, pp 301–315. https://doi.org/10.1016/j.ymeth.2012.12.005.

(4) Gustafsson, N.; Culley, S.; Ashdown, G.; Owen, D. M.; Pereira, P. M.; Henriques, R. Fast Live-Cell Conventional Fluorophore Nanoscopy with ImageJ through Super-Resolution Radial Fluctuations. *Nat. Commun. 2016 71* **2016**, *7* (1), 1–9. https://doi.org/10.1038/ncomms12471.

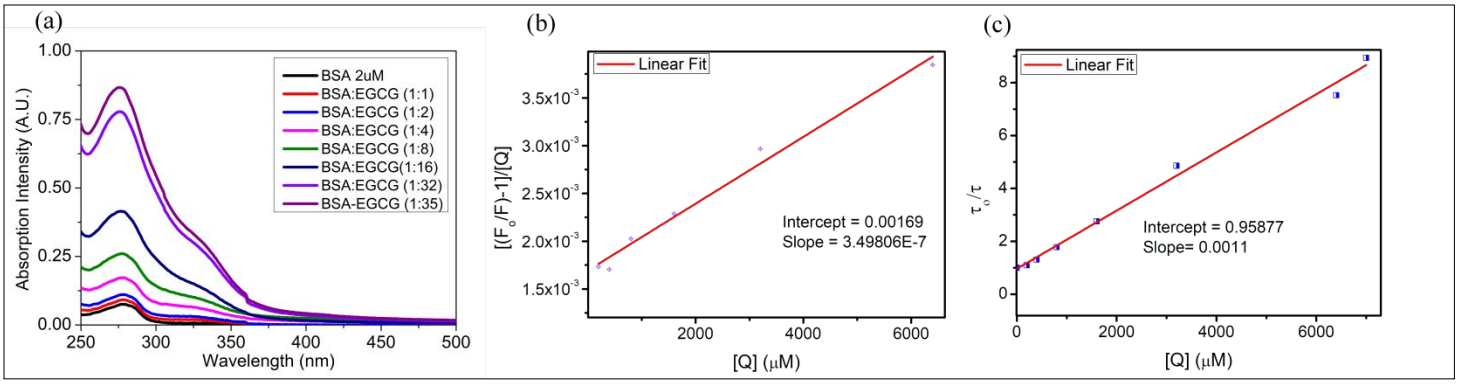

Figure-S1: (a) UV absorption spectra of BSA and its conjugation with EGCG in increasing ratio. (b) Fitted plot between $\frac{1}{[Q]}\left[\frac{F_0}{F}-1\right]$ vs $[Q]$ for simultaneous static and dynamic quenching. (c) Fitted plot between $\frac{t_0}{t}$ vs $[Q]$ for estimation of dynamic quenching constant K_D.

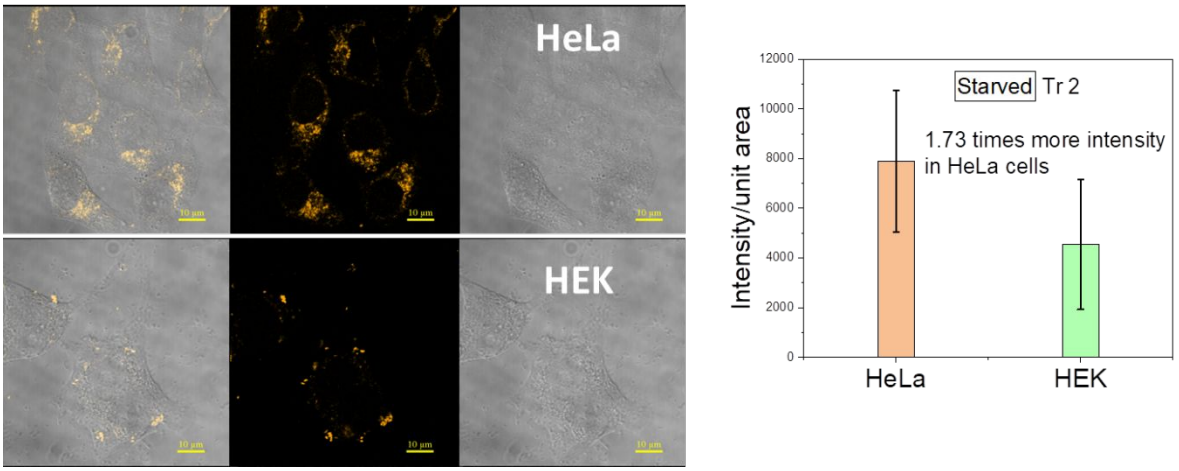

Figure S2: Confocal images and comparison of HeLa cells and HEK-293 cells treated with BSA conjugated EGCG.

Table S1: Average Lifetime of BSA-EGCG conjugation after fitting into the appropriate exponential.

Sample	T1(ns)	T2(ns)	T3(ns)	Tavr (ns)
BSA	5.23(50.11%)	7.54(49.89%)		**6.17**
BSA-EGCG (1:1)	3.34(17.68%)	6.66(82.32%)		**5.66**

BSA-EGCG (1:2)	2.13(16.89%)	6.30(83.11%)		**4.73**
BSA-EGCG (1:4)	3.11(27.39%)	6.48(66.24%)	0.65 (6.37%)	**3.47**
BSA-EGCG (1:8)	2.71(32.95%)	6.32(55.11%)	0.51(11.94%)	**2.24**
BSA-EGCG (1:16)	2.09(36.37%)	5.85(43.47%)	0.37(20.16%)	**1.27**
BSA-EGCG (1:32)	1.79(38.61%)	5.42 (32.13%)	0.31(29.26%)	**0.82**
BSA-EGCG (1:35)	1.72(38.82%)	5.24(27.81%)	0.28(33.37%)	**0.69**

Table-S2: Proportion of secondary structure change upon binding of Ligand EGCG to BSA.

S. No.	Sample types	%Helix	change in % Helix
1	BSA	47.98	None
2	BSA-EGCG	61.01	27.15

Table-S3: Viscosity-dependent lifetime change of BSA-Cy3 in PBS solution phase.

Sample	**BSA**	**BSA-EGCG**		
		1:02	**1:35**	**2:70**
pH 4.3 (50% Glycerol)	**3.5**			
pH 5.3 (50% Glycerol)		**3.5**	**3.5**	**3.2**

pH 5.3 (25% Glycerol)		**3.5**	**3.2**	**3**
pH 5.3 (0% Glycerol)	**2.7**	**2.9**	**2.8**	**2.5**