Electronic Supplementary Information
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Functionalized Chitosan with Self-assembly-Induced and Subcellular Localization-Dependent Fluorescence 'Switch On' Property

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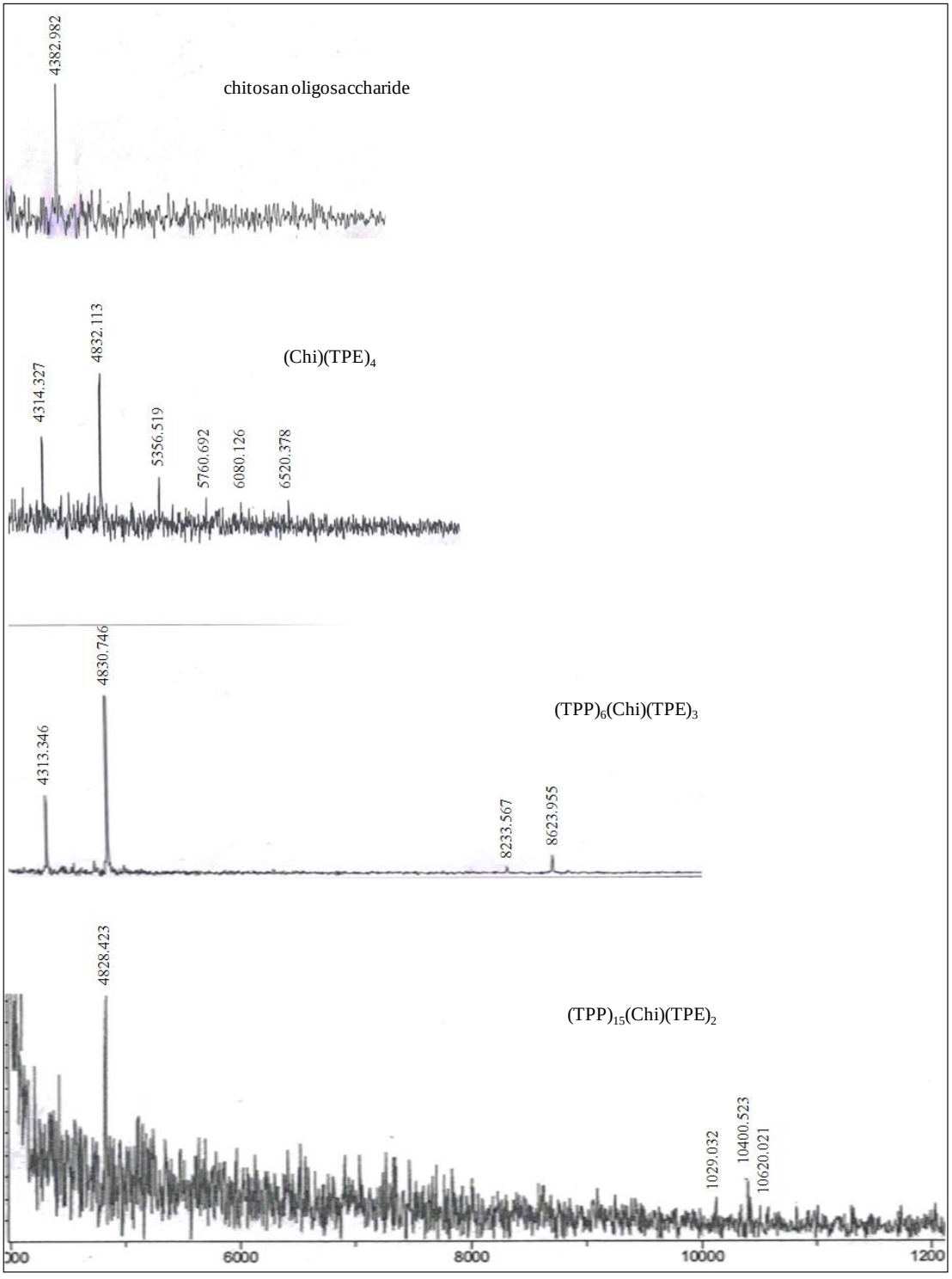

Figure S1. MALDI-TOF-MS of chitosan oligosaccharide, $(Chi)(TPE)_4$, $(TPP)_6(Chi)(TPE)_3$ and $(TPP)_{15}(Chi)(TPE)_2$ using DHB (2,4-dihydroxy benzoic acid) matrix. Chitosan shows peak at 4382.982. Peak at 6080.126, 6520.378 suggest formation of $(Chi)(TPE)_4$. Peak at 8233.567, 8623.955 suggest formation of $(TPP)_6(Chi)(TPE)_3$. Peak at 1029.032, 10400.523 suggest formation of $(TPP)_{15}(Chi)(TPE)_2$.

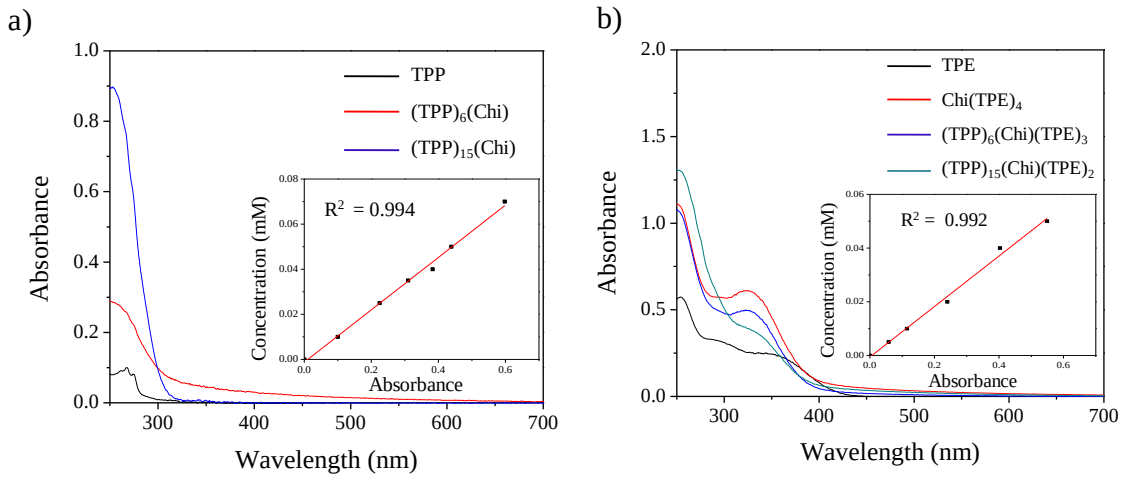

Figure S2. Preparation of calibration graphs for calculating number of TPP/TPE per chitosan. a) Typical absorption spectra of TPP, $(TPP)_6(Chi)$ and $(TPP)_{15}(Chi)$ and plot of absorbance at 267 nm against the concentration in the inset. b) Typical absorption spectra of TPE, $Chi(TPE)_4$, $(TPP)_6(Chi)(TPE)_3$ and $(TPP)_{15}(Chi)(TPE)_2$ and plot of absorbance at 354 nm against the concentration of TPE.

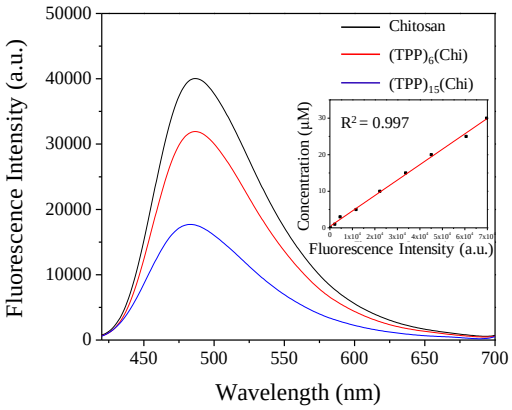

Figure S3. Quantification of number of TPP in functional chitosan by determining primary amines in chitosan oligosaccharide via fluorescamine test. Typically, excess fluorescamine is reacted with functional chitosan and the fluorescence intensity at 484 nm is used to estimate the primary amines. Emission spectra are shown for various functional chitosan after reaction with fluorescamine. First, excess fluorescamine is reacted with varying concentration of functional chitosan oligosaccharide and a standard calibration is prepared by measuring fluorescence intensity at 484 nm against the concentration of chitosan oligosaccharide. (shown in the inset) Next, this calibration graph is used to estimate the amines in functional chitosan.

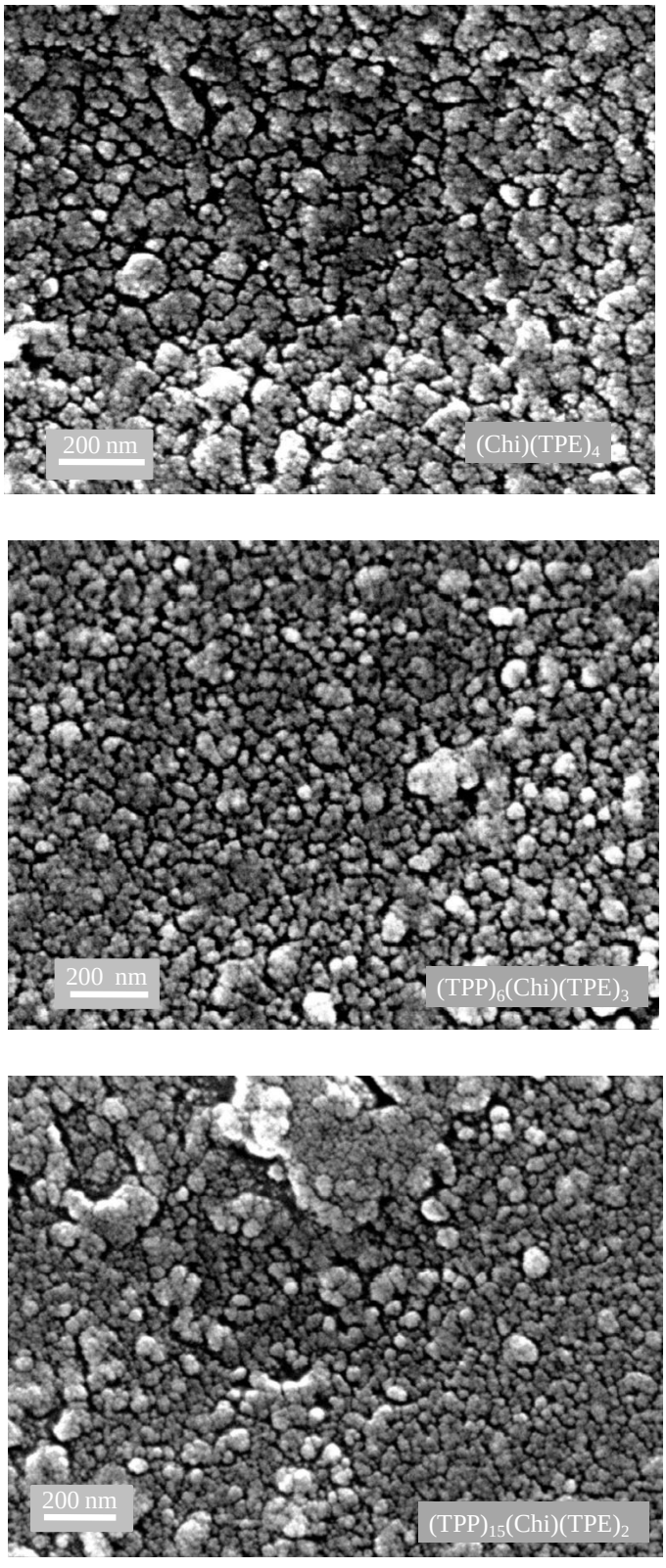

Figure S4. SEM image of self-assembled functional chitosan oligosaccharides, showing their size in the range of 50-200 nm.

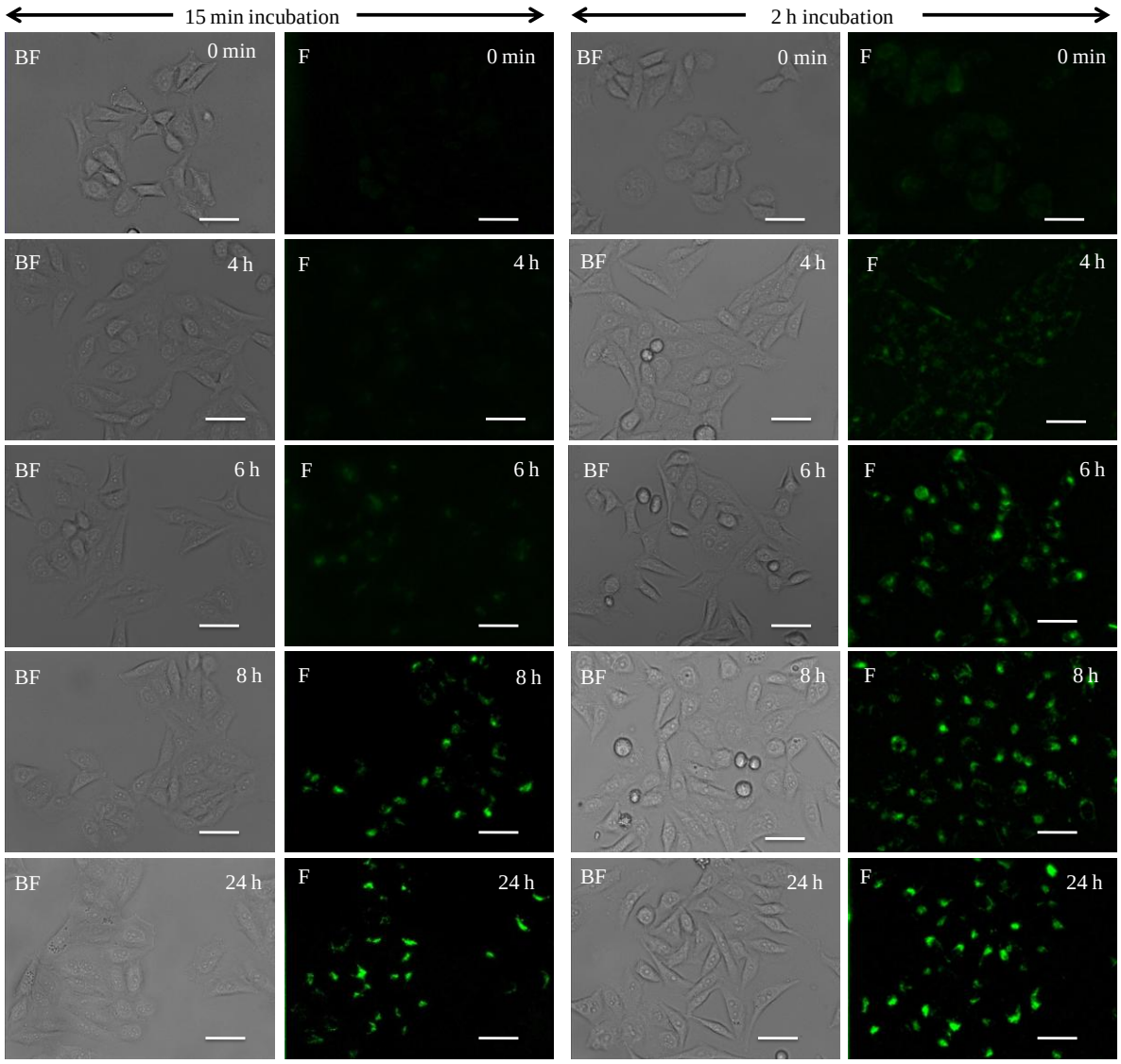

Figure S5. Lower magnification image of cell labeling and subcellular localization dependent fluorescence 'switch on' property of $(TPP)_6(Chi)(TPE)_3$. HeLa cells were incubated with sample for 15 min or 2 h and then washed cells are incubated with fresh culture media upto 24 h and imaged under bright field (BF) and fluorescence (F) mode. Results show appearance of fluorescence and gradual increase of fluorescence intensity with increasing incubation time followed by localization of fluorescence spots inside cytoplasm. Scale bar represents 50 microns.

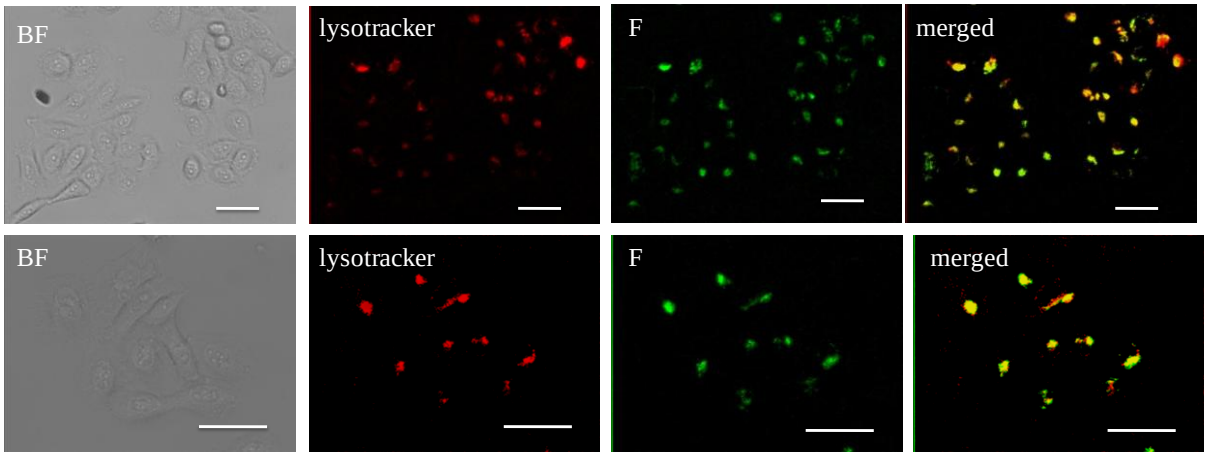

Figure S6. Additional data on colocalization study of $(TPP)_6(Chi)(TPE)_3$ with lysotracker red in HeLa cell showing that $(TPP)_6(Chi)(TPE)_3$ localize in lysozome. Typically, cells are incubated with sample for 2 h and then washed cells are incubated with fresh culture media for 24 h followed by incubation with lysotracker for 30 min. Next, washed cells are imaged under bright field (BF) and fluorescence (F) mode. Merged yellow image shows significant colocalization of $(TPP)_6(Chi)(TPE)_3$ with lysotracker. Scale bar represents 50 microns.

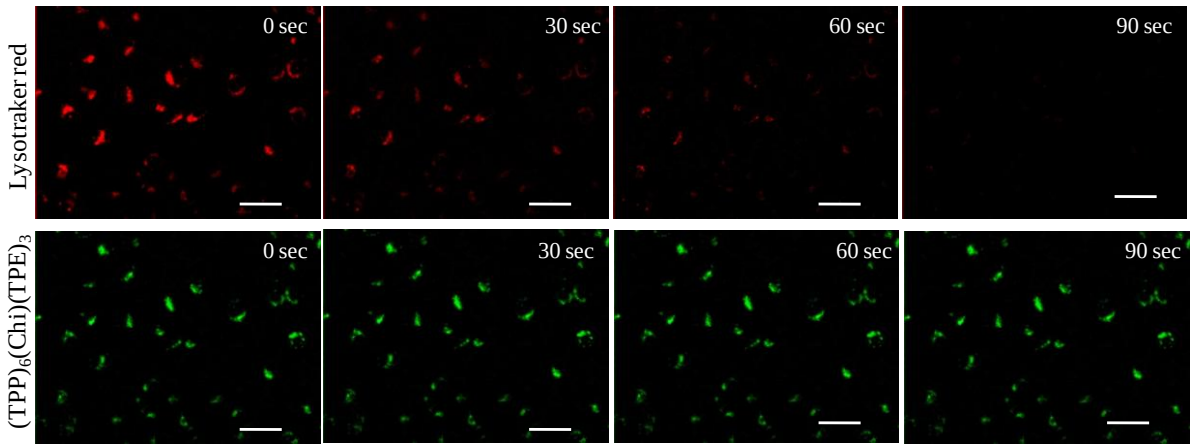

Figure S7. Additional data on photostability of $(TPP)_6(Chi)(TPE)_3$ over lysotracker red. Labelled HeLa cells are continuously exposed with light and imaged after 0 second, 30 seconds, 60 seconds and 90 seconds. Green excitation (480-550 nm) is used for imaging of lysotracker red and UV excitation (330-390 nm) is used for imaging of $(TPP)_6(Chi)(TPE)_3$. Results show completely quenched fluorescence of lysotracker red within 60 sec due to photobleaching effect but fluorescence of $(TPP)_6(Chi)(TPE)_3$ remain stable. Scale bar represents 50 microns.

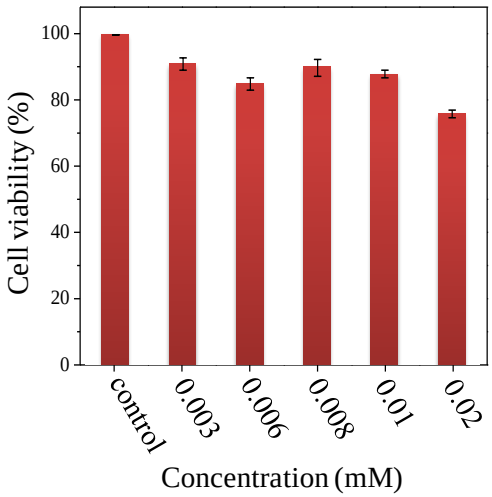

Figure S8. Cytotoxicity of $(TPP)_6(Chi)(TPE)_3$ measured via MTT assay. Typically, HeLa cells are incubated with $(TPP)_6(Chi)(TPE)_3$for 24 h and then used for cytotoxicity study. Cell viability has been determined by assuming 100 % viability for control sample without $(TPP)_6(Chi)(TPE)_3$. The mean ± SD of three determination (n=3) are represented in bars. The mean ± SD of three determination (n=3) are represented in bars.

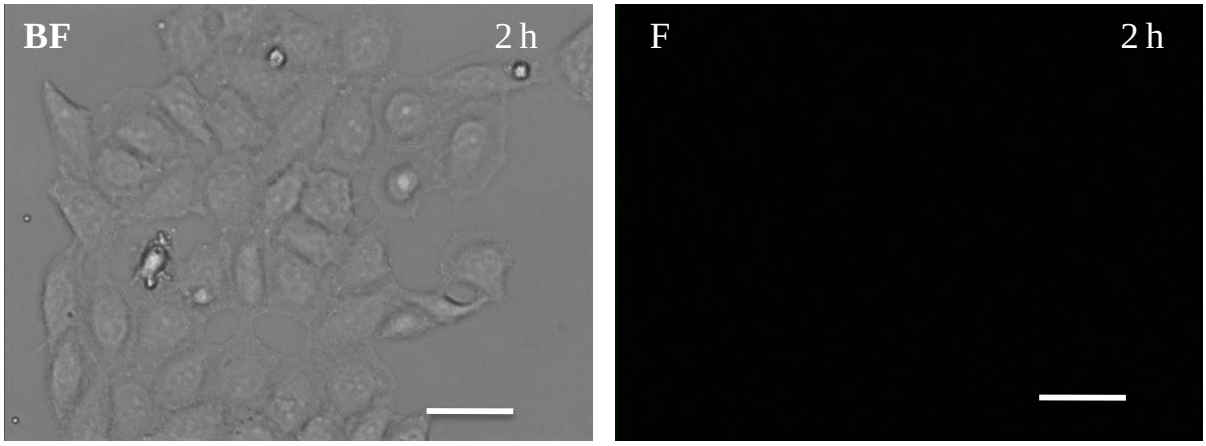

Figure S9. Energy dependent internalization study of $(TPP)_6(Chi)(TPE)_3$. Cells are incubated with $(TPP)_6(Chi)(TPE)_3$ at 4 °C for 2 h, washed with PBS and fresh media is added and imaging is done after 8 h under UV excitation. No labeling of cells suggest poor endocytotic uptake of $(TPP)_6(Chi)(TPE)_3$. Scale bar represents 50 microns.

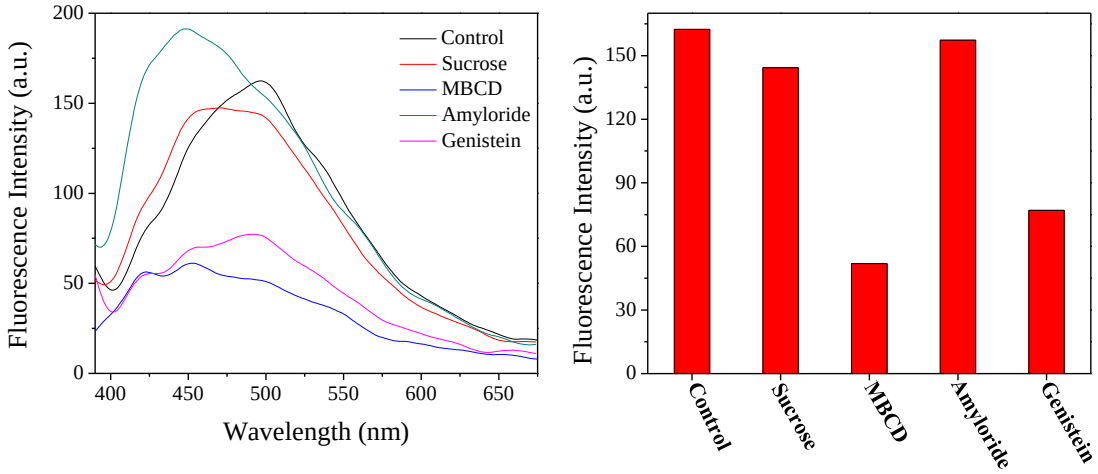

Figure S10. Quantitative estimation of cellular uptake of $(TPP)_6(Chi)(TPE)_3$ in HeLa cells in the presence of different endocytosis inhibitors via fluorescence measurement using fluorescence plate reader. Typically, cells are incubated with inhibitors followed by incubation with samples and after 1 h washed cells are used for fluorescence measurement in fluorescence plate reader (a) and quantified using fluorescence intensity at 480 nm (b). As inhibitors are toxic for long time cell survival but longer time is required for fluorescence 'switch on' effect, we have used high concentration (12 μM) of $(TPP)_6(Chi)(TPE)_3$ to ensure fluorescence within 1 h and before appreciable cytotoxicity. Results show that cellular uptake is predominantly inhibited by both genistein and methyl-β-cyclodextrin (MBCD).

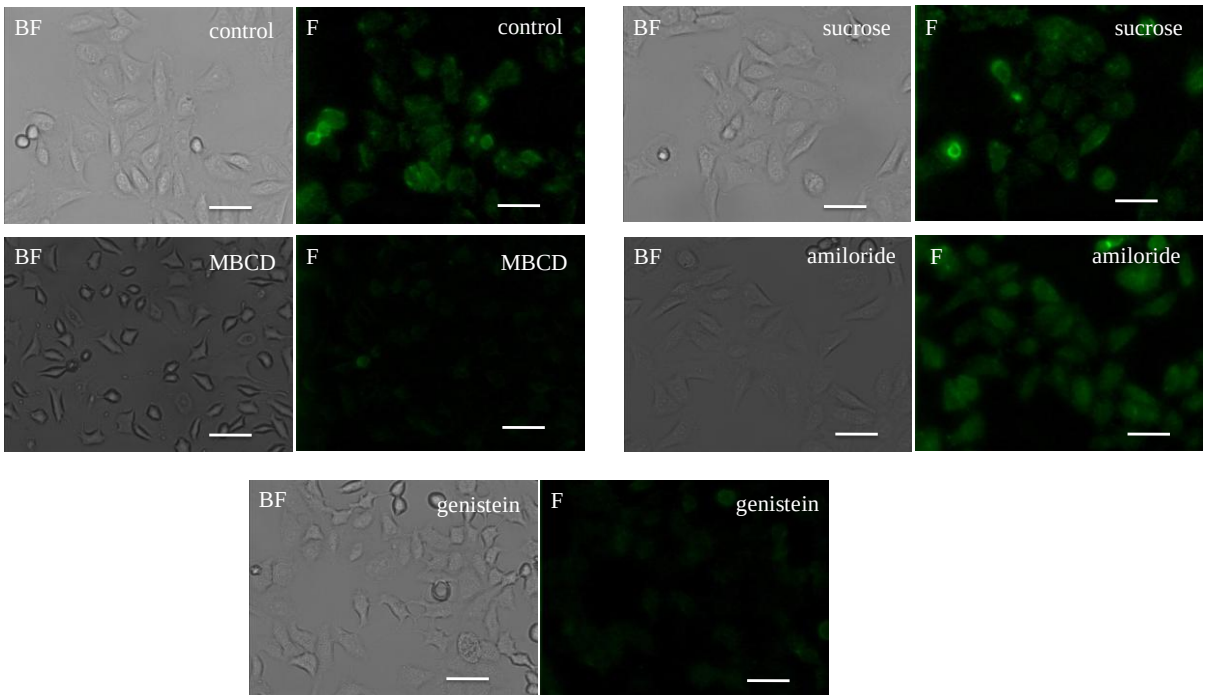

Figure S11. Typical fluorescence image of HeLa cells labelled with $(TPP)_6(Chi)(TPE)_3$ in the presence of different endocytosis inhibitors, that is used for measuring quantitative cell uptake (shown in Figure S10). Typically, cells are incubated with inhibitors for 1 h followed by incubation with $(TPP)_6(Chi)(TPE)_3$, and washed cells are imaged under the fluorescence microscope. Images show that cellular uptake is predominantly inhibited by both genistein and methyl-β-cyclodextrin (MBCD).

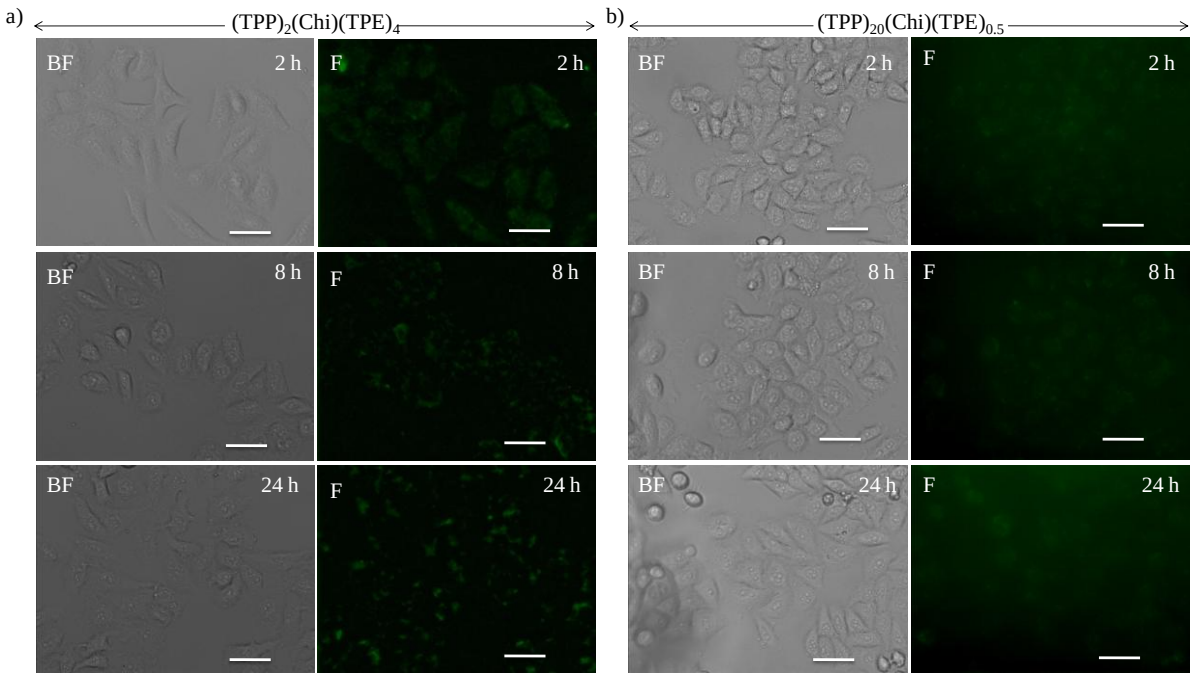

Figure S12. Lower magnification image of HeLa cells labeled with a) $(TPP)_2(Chi)(TPE)_4$ and b) $(TPP)_{20}(Chi)(TPE)_{0.5}$. Cells are incubated with sample for 2 h and then washed cells are incubated with fresh culture media upto 24 h and imaged under bright field (BF) and fluorescence (F) mode. Results show that $(TPP)_2(Chi)(TPE)_4$ have low cell entry and stack on cell membrane surface and $(TPP)_{20}(Chi)(TPE)_{0.5}$ show very low fluorescence intensity after cell entry. Scale bar represents 50 microns.

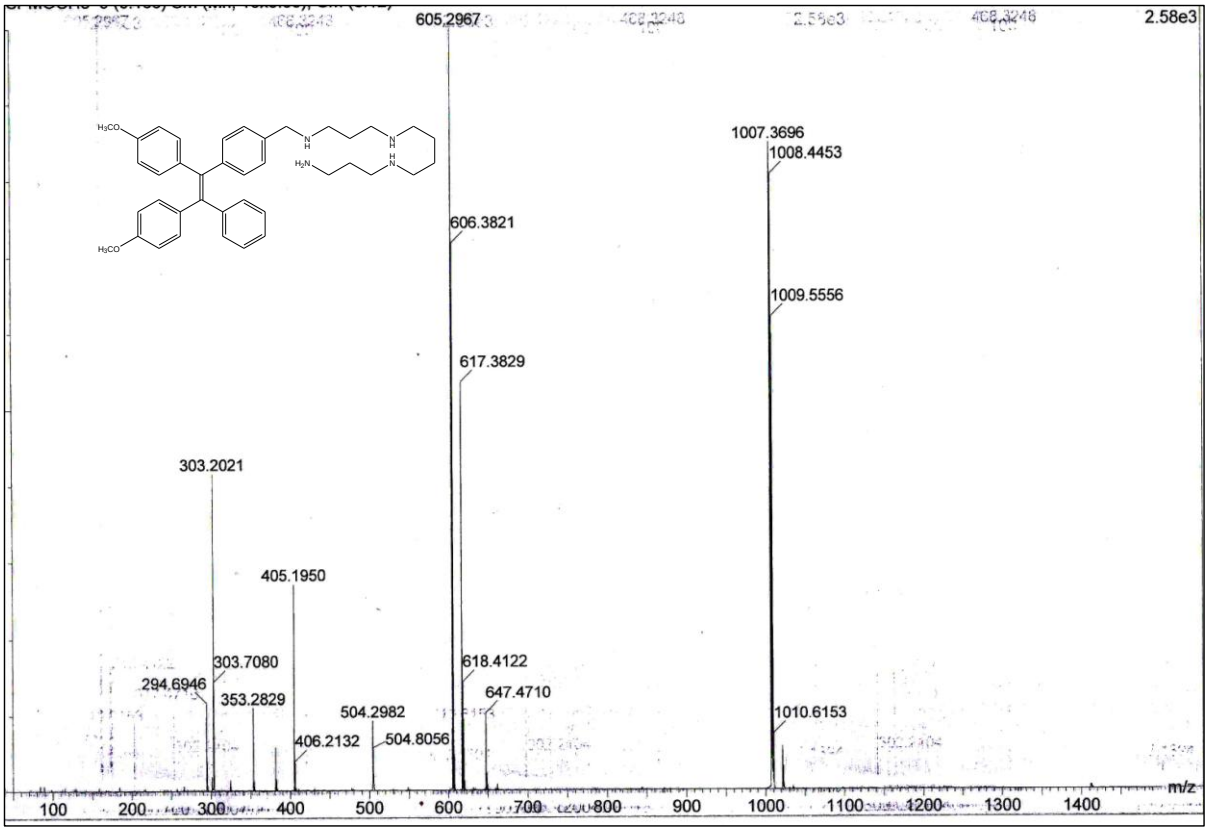

Figure S13. HRMS of TPE conjugated spermine. The peak at 606.3821 suggests the formation of conjugate. (calculated for $C_{39}H_{49}N_4O_2$ (M^+): 606.3934; found 606.3821). The peak at 1008.4453 suggests the formation of two TPE conjugated spermine as side product. (calculated for $C_{68}H_{72}N_4O_4$ (M^+-2H): 1008.6; found 1008.4453).

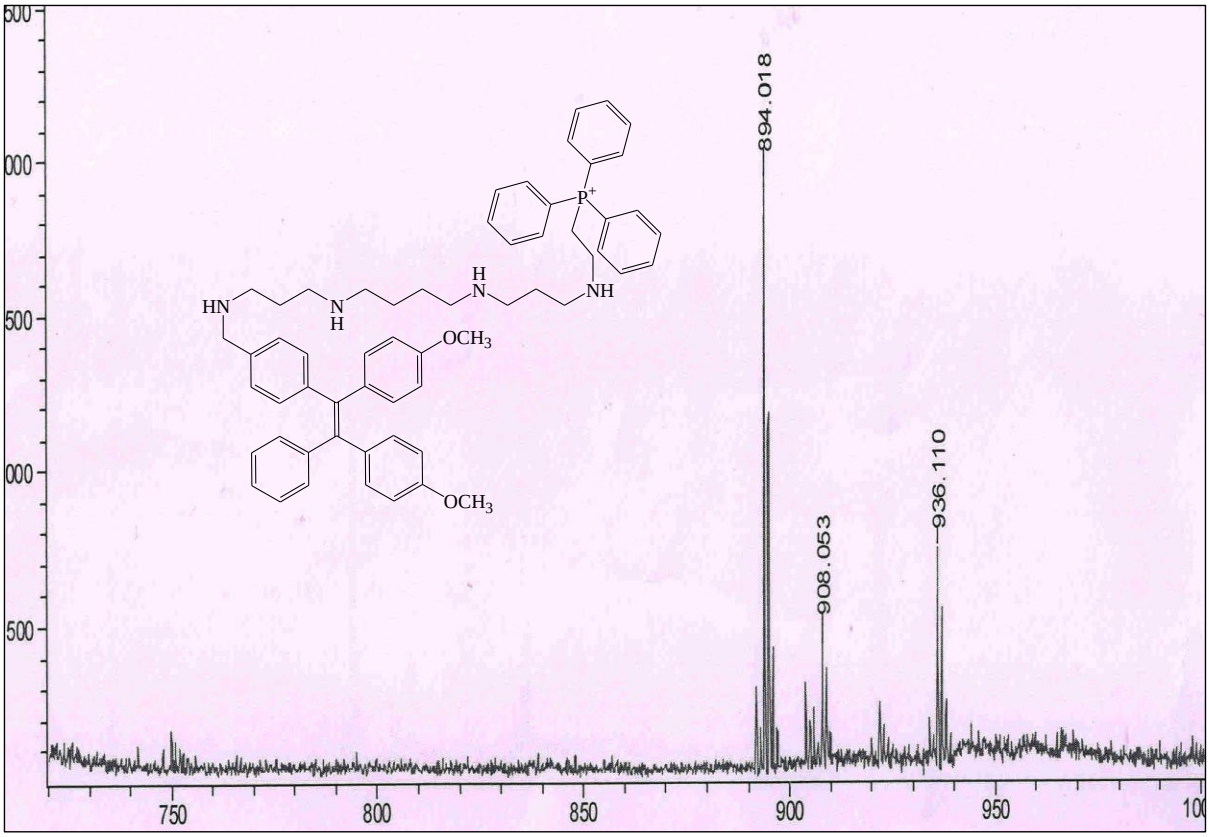

Figure S14. HRMS of TPE and TPP conjugated spermine. The peak at 894.018 suggest the formation of conjugate (calculated for $C_{59}H_{67}N_4O_2P^+$ (M^+-H): 894.5; found 894.018)

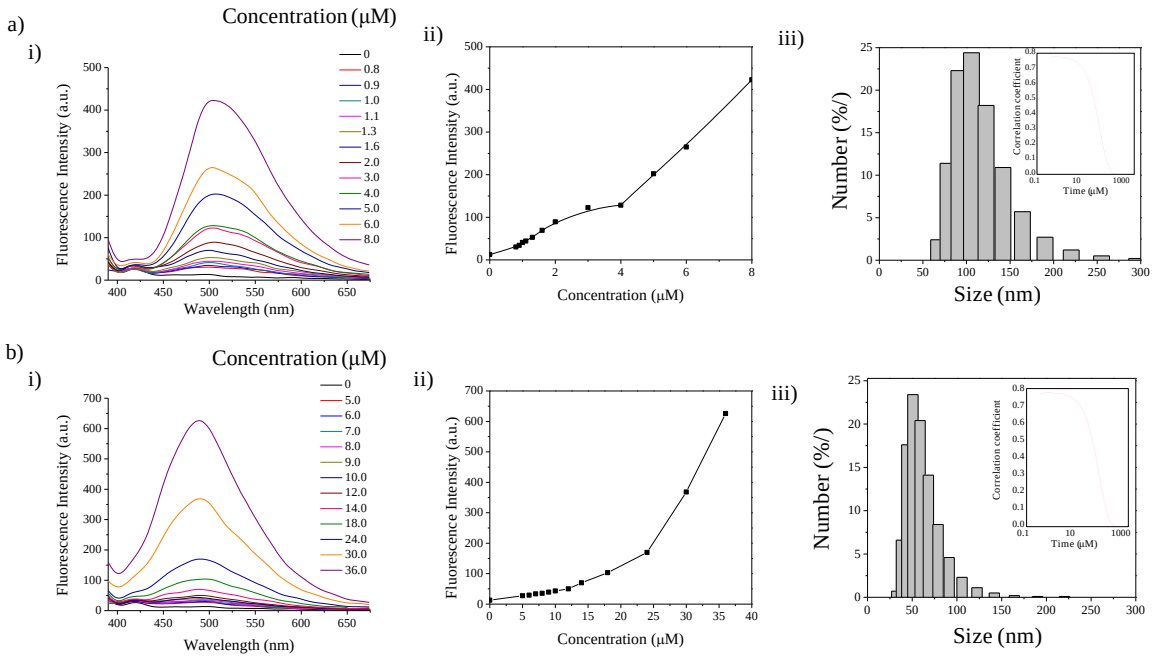

Figure S15. a) i) Concentration dependent fluorescence spectra of TPE functionalized spermine in 80 % water-DMSO under 370 nm excitation and ii) plot of emission intensity at 504 nm against concentration, showing CAC at ~ 4.0 μM. iii) hydrodynamic size above the CAC as observed by DLS.

b) i) Concentration dependent fluorescence spectra of TPE and TPP functionalized spermine in 80 % water-DMSO under 370 nm excitation ii) plot of emission intensity at 489 nm against concentration showing CAC at ~ 24 μM. iii) hydrodynamic size above the CAC as observed by DLS.

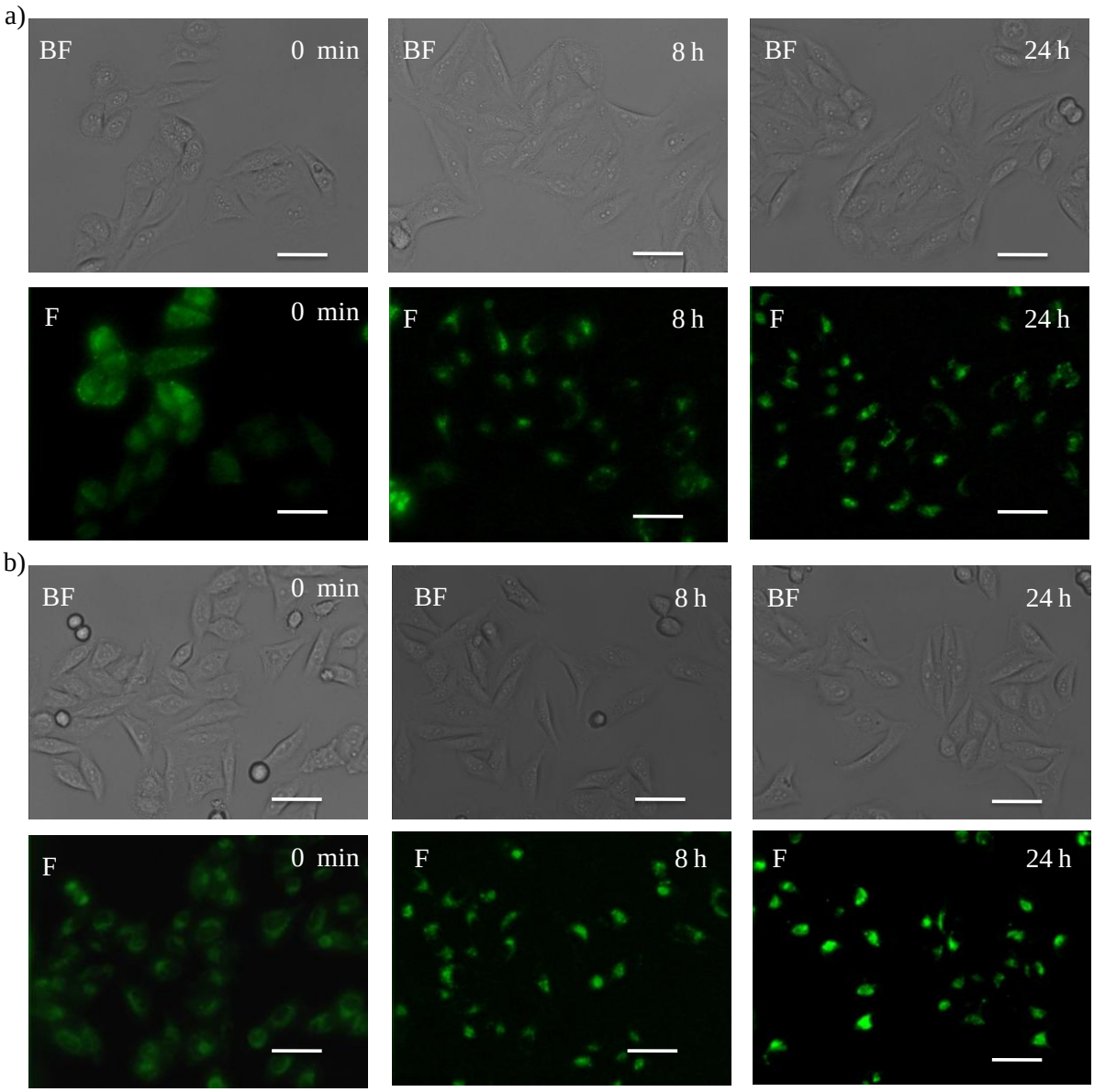

Figure S16. Loss of subcellular localization dependent fluorescence 'switch on' property by a) TPE functionalized spermine and b) TPE and TPP functionalized spermine. HeLa cells are incubated with DMSO solution of sample 2 h and then washed cells are incubated with fresh culture media upto 24 h and imaged under bright field (BF) and fluorescence (F) mode. Results show fluorescence immediately after labelling that increases with incubation time and appears poor localization inside cytoplasm. Scale bar represents 50 microns.