Electronic Supplementary Information

EXPERIMENTAL SECTION

Chemicals. L-ascorbic acid, glutaraldehyde, L-histidine, methyl glyoxal, triethyl amine (Et_3N), Dulbecco's modified eagle medium (DMEM), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), diethyl ether, potassium iodide, nile red and starch were purchased from Sigma-Aldrich. Methyl-thiazolyl-diphenyltetrazolium bromide (MTT), penicillin−streptomycin antibiotic and fetal bovine serum (FBS) were purchased from Hi-Media.

Instrumentation. Transmission electron microscopic (TEM) images were recorded using an ultra-high-resolution field emission gun transmission electron microscope (JEOL JEM-2100 PLUS) with a 200kV electron source. Hydrodynamic size and zeta potential were measured using a Malvern Nano ZS instrument. In vitro cell imaging was carried out using an IX81 microscope with CellSense software. Mass spectra of samples were collected using a Quadruple TOF (Q-TOF) micro-MS system using the electrospray ionization (ESI) (via a Thermo Scientific LTQ Orbitrap XL hybrid Fourier transform mass spectrometer). MALDI-TOF experiment were performed using an Ultraflextreme mass spectrometer (Bruker Daltonics) equipped with a Bruker smart beam II 355 nm nitrogen laser with dithranol as a matrix. Nuclear magnetic resonance (NMR) spectra were captured by a Bruker DPX-400 using D_2O as a solvent. Fourier transform infrared (FTIR) spectra were recorded with a Nicolet 6700FTIR instrument (Thermo Scientific) using KBr pellets. UV−visible absorption spectra were collected using a multimode ultraviolet microplate reader (Molecular Devices, SpectraMax M2e) and Shimadzu UV−visible spectrometer. UV−visible absorption spectra of samples were recorded using a Shimadzu UV-2550 UV−visible spectrophotometer.

Synthesis of vitamin C nanoparticles. For the synthesis of vitamin C nanoparticles, 47 mg (0.27 mM) of vitamin C was dissolved in 10 mL of ethanol-water solvent mixture (ethanol: water as 4:1) at room temperature, followed by addition of 15 μL (0.135 mM) of glutaraldehyde or 20 μL (0.27 mM) of methylglyoxal. Then, 42 mg (0.27 mM) of L-histidine was added into the solution and stirring was continued for overnight. We have used two different di-carbonyls such as glutaraldehyde or methyl glyoxal that produces compound I and compound II, respectively. The ethanol–water ratio (4:1) was selected based on its ability to dissolve all three reactant molecules. This homogeneity was critical to facilitate efficient interaction among the reactants and ensure successful synthesis of both compound I and compound II. Next, both the synthesized compounds were precipitated by adding excess acetone. After that, precipitate was cleaned with diethyl ether twice and dried under vacuum. About 40 mg of the material was obtained. Finally, compounds precipitate was redispersed in 1.5 mL milli Q water (~27 mg/mL) and stored at 4°C. The compounds were characterized by HRMS, MALDI-TOF mass spectrometry, ^{1}H NMR and FTIR.

Characterization of the nanoparticles. The stability of nanoparticles I was checked by keeping their solution in different pH (4.6, 7.4 , 9) 24 hours and TEM size measurement was performed. For quantification of the vitamin C, the nanoparticle I solution was diluted with water, and then, absorbance was measured using a UV−visible spectrophotometer to estimate the concentration of the vitamin C. For release profile of the vitamin C from nanoparticle solution was kept at pH 4.6 inside a dialysis bag (MW cut off 12 kDa) and solution was dialyzed extensively against water in physiological pH condition. After different time interval, water solution outside was used to measure the amount of vitamin C released from the absorbance of vitamin C at 265 nm.

Determination of hydrogen peroxide (H_2O_2). The starch-iodine colorimetric titration method was applied to determine hydrogen peroxide in the nanoparticle. In brief, nanoparticle I (vitamin C concentration = 70 mM) was kept in acidic pH (4.6) solution for 4h to release vitamin C in molecular form. Next, the solution pH was adjusted with physiological pH of 7.4 by the addition of NaOH. Next, KI (200 mM) and starch (6 mM) were added to the solution mixture. Then, this mixture was taken in a 15 mL vial, kept under the dark and purged with pure oxygen. At different time interval, UV−visible absorption spectra were measured to monitor generation of hydrogen peroxide. Appearance of absorbance band at 575–600 nm range indicate presence of hydrogen peroxide. After the release of molecular vitamin C from nanoparticle I, we varied the solution pH to investigate hydrogen peroxide production under similar experimental conditions.

ROS detection using dye-based degradation assay. The rhodamine B dye degradation method was employed to detect ROS generated from the nanoparticle system. Briefly, nanoparticle I (containing 70 mM vitamin C) was incubated in an acidic solution (pH 4.6) for 4 hours to facilitate the release of vitamin C in its molecular form. Subsequently, the pH of the solution was adjusted to physiological pH (7.4) by the addition of NaOH. Rhodamine B (5 μM) was then added to the mixture. The resulting solution was transferred to a 15 mL vial, kept in the dark, and purged with pure oxygen. UV−visible absorption spectra were recorded at 550 nm at various time intervals to monitor ROS generation through dye degradation.

Determination of vitamin C concentration inside cell. KB cells were cultured in a 25 cm^2 cell culture flask under 37 °C with 5% CO_2 in DMEM containing 10% heat-activated fetal bovine serum (FBS) and 1% penicillin−streptomycin antibiotic. Cells were subcultured in a 24-well plate containing DMEM for 24h until cells were adhered. Next, cells were taken in 500 μL of fresh DMEM and treated with final nanoparticle concentration of 1.45 mg/mL (with vitamin C concentration of 2 mM). Next, cells were incubated with nanoparticle for 10 min followed by washing with HEPES buffer solution to remove unbound nanoparticle and fresh media were added for further 2h incubation. Finally, cells were tripsinized and collected in one mL PBS buffer for UV−visible absorption spectra. Cells without treatment of any sample were considered as a control.

For the subcellular localization study, KB cells were first treated with lysotracker blue for 2 hours. This was followed by the incubation of a nile red–incorporated vitamin C nanoparticle solution (containing 2 mM vitamin C), and the cells were incubated for 10 minutes at 37 °C. After incubation, the cells were washed and imaged using a confocal microscope.

For intracellular ROS generation study, KB cells were treated with vitamin C nanoparticles for 2h, followed by addition of 3 μL DCFH-DA solution (2 mM) and incubated for 20 min at 37 °C. Next, cells were washed and imaged under fluorescence microscope.

For intracellular superoxide radical detection, KB cells were treated with vitamin C nanoparticles for 2h, followed by addition of 500 μL HEPES buffer solution of MitoSOX (2 μM) and incubated for 30 min at 37 °C. Next, cells were washed and imaged under fluorescence microscope.

Cytotoxicity study. KB cells were cultured in a 24-well plate for 24 h. After that, cells were washed with HEPES buffer solution and 500 μL of fresh DMEM media was added. Next, different doses of nanoparticle were added and incubated for 24h. Then, cells were washed with HEPES buffer at pH 7 and DMEM media was added. Then, 40 μL of freshly prepared MTT (5 mg/mL) solution was added to each cell for 4-5 h. Next, the supernatant was carefully removed, and the formed violet formazan precipitate was dissolved by 600 μL of sodium dodecyl sulfate solution (DMF: water = 1:1). At last, the absorbance was recorded using a plate reader at 570 nm. The cells without treatment of any sample were referred as controls with 100 % cell viability.

Statistical analysis. Each data was represented as mean ± SD. One-way analysis of variance (ANOVA) and two-way analysis of variance (ANOVA) were applied for multigroup comparisons. All statistical analyses were done using GraphPad Prism 8.0.2software. Statistical significance in figures was considered as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, and n.s (non-significant).

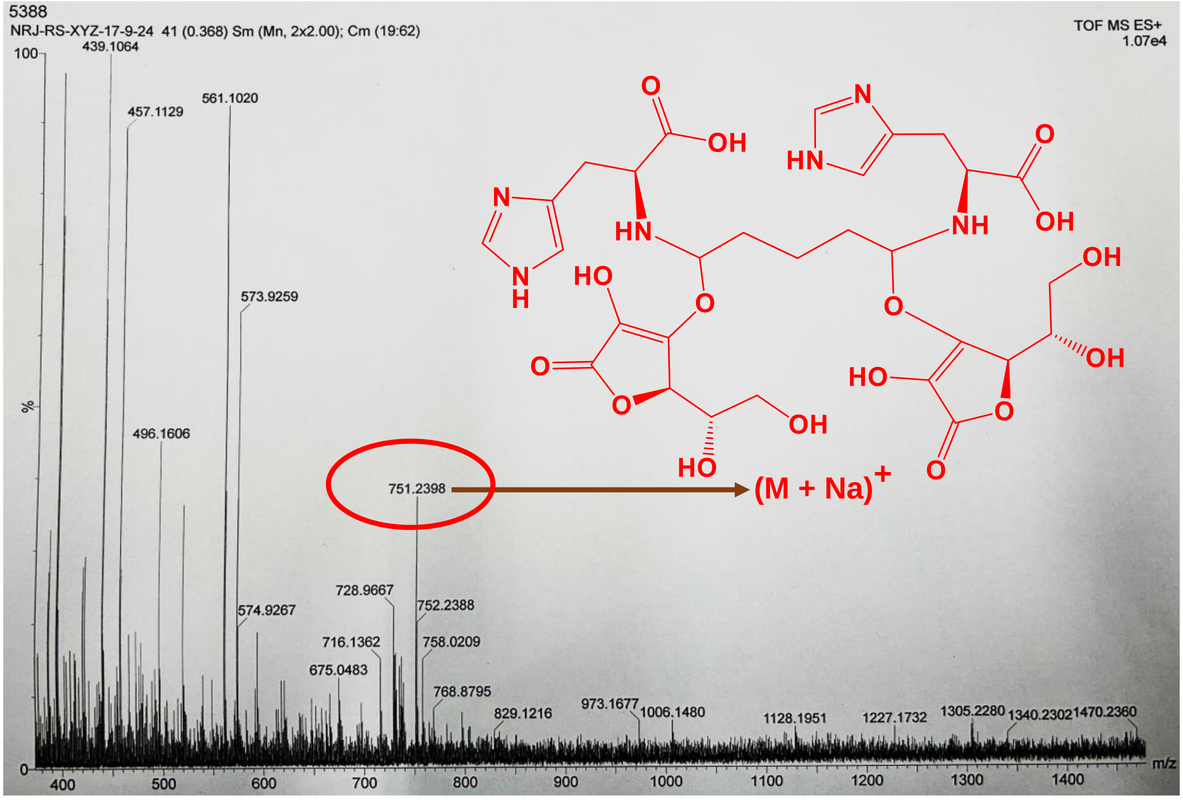


Figure S1. HRMS of compound I. The peak at 751.24 corresponds M+ Na^{+}.

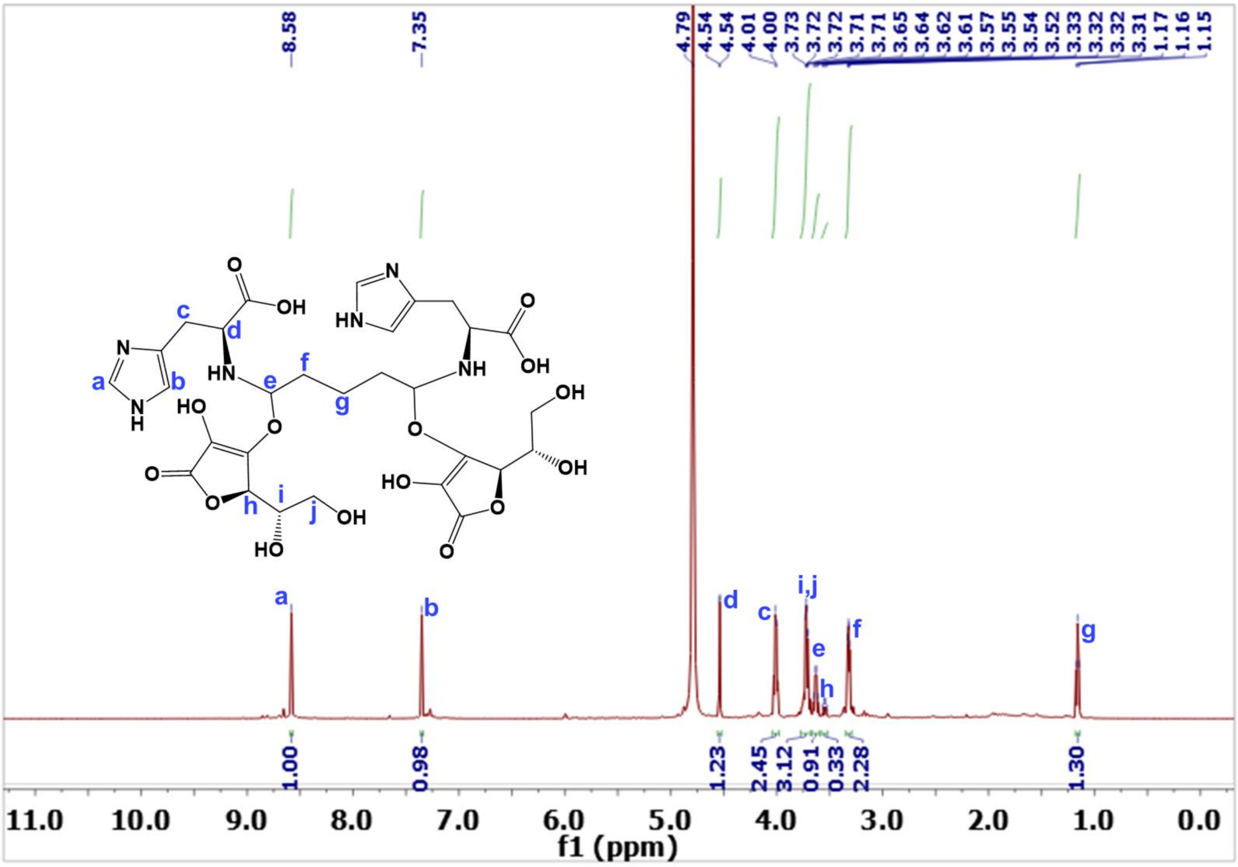


Figure S2. [1]H NMR characterization of compound I using D_2O as solvent.

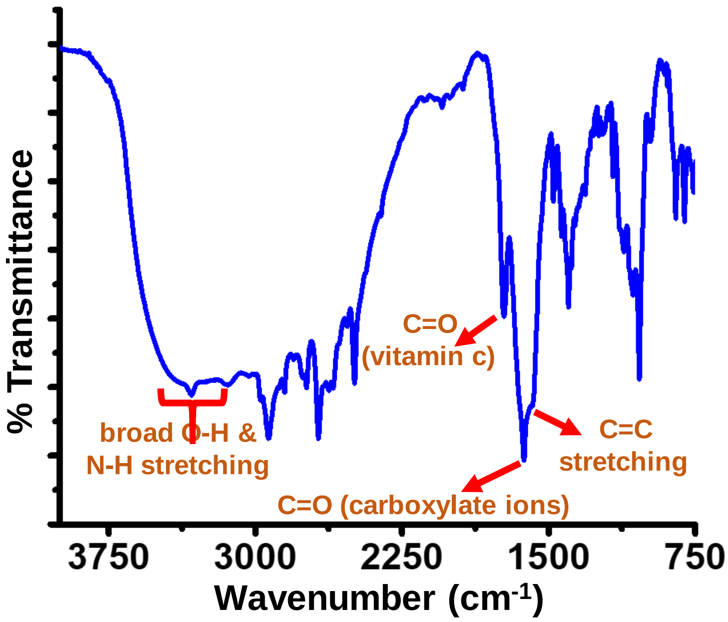


Figure S3. FTIR spectrum of compound I showing the characteristic stretching frequencies of different functional groups.

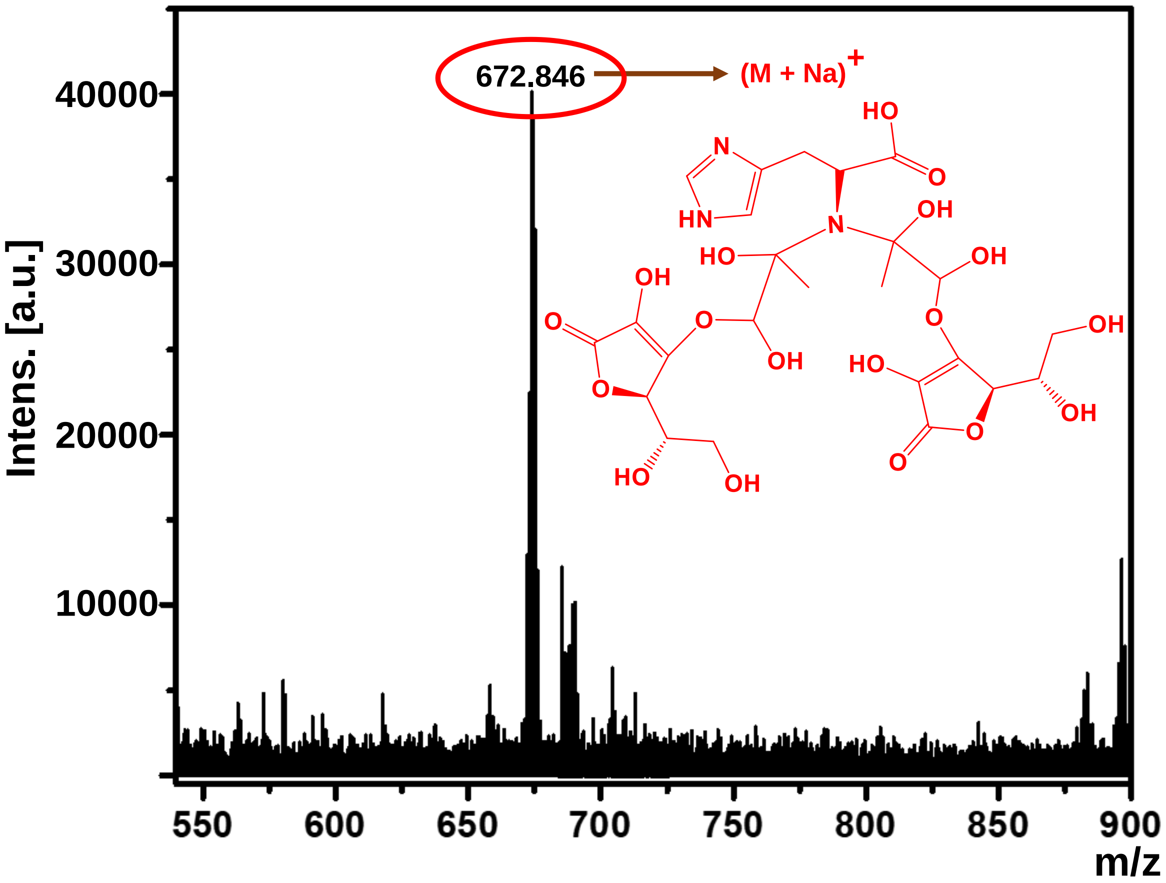


Figure S4. MALDI mass spectrum of compound II using dithranol as matrix. The peak at 672.85 corresponds M^+ Na^+.

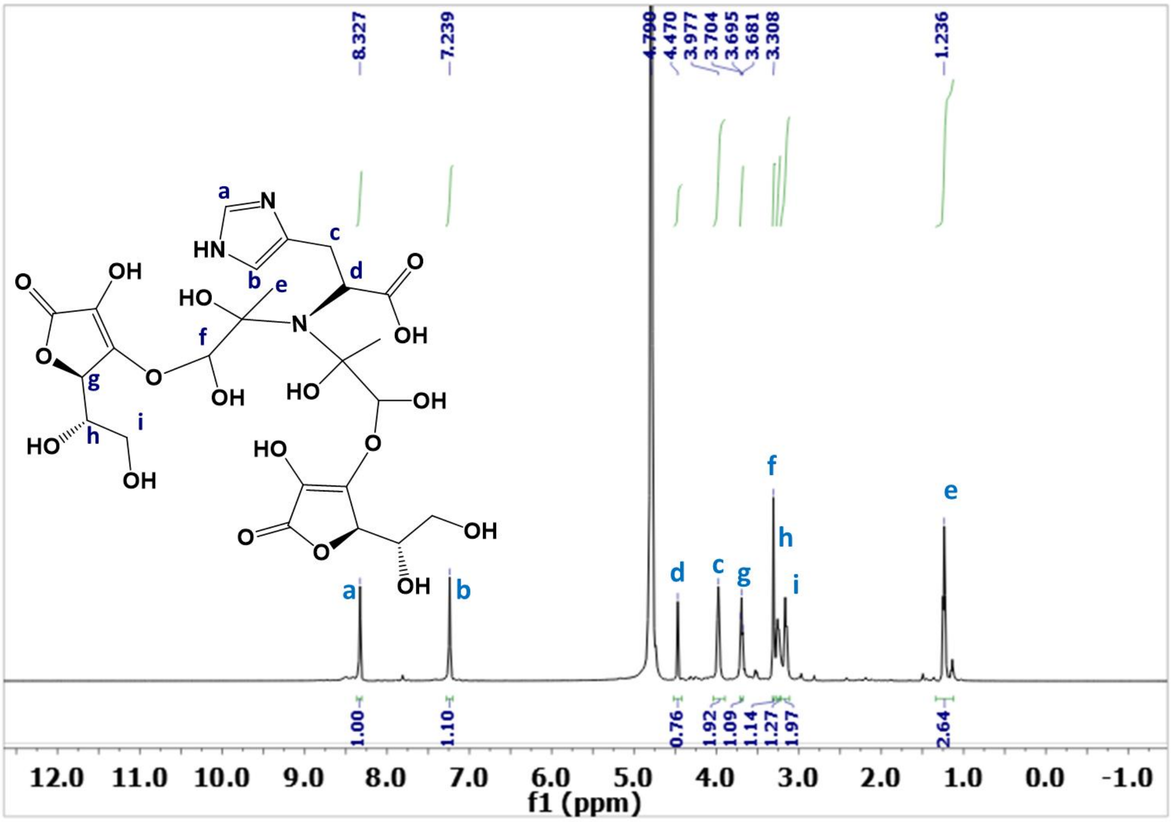


Figure S5. [1]H NMR characterization of compound II using D_2O as solvent.

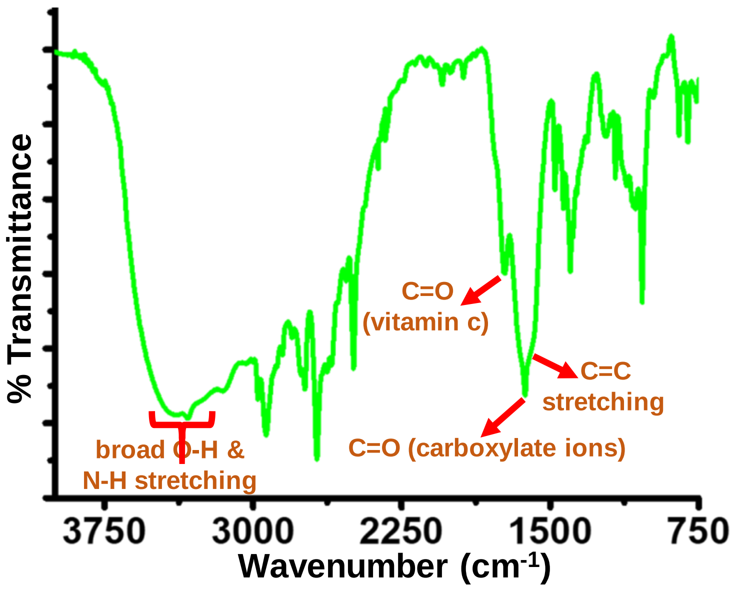
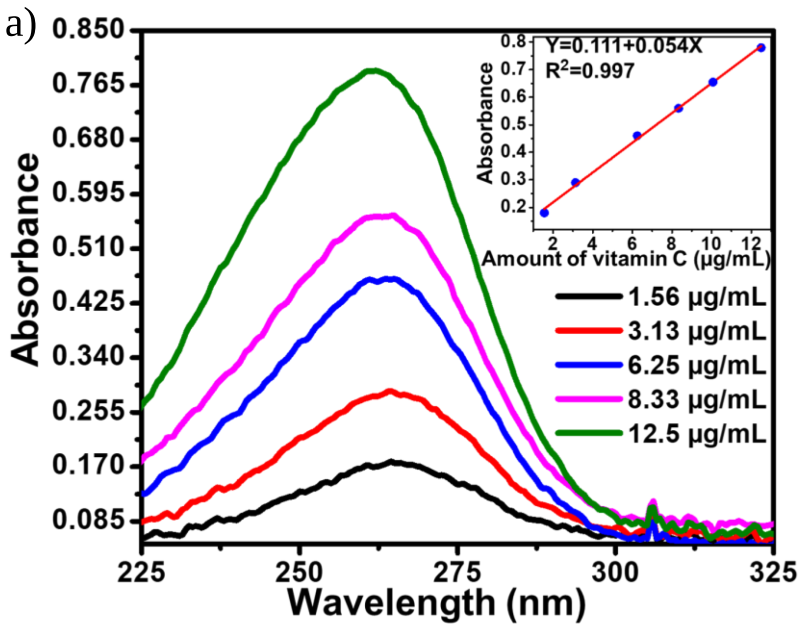


Figure S6. FTIR spectrum of compound II showing the characteristic stretching frequencies of different functional groups.



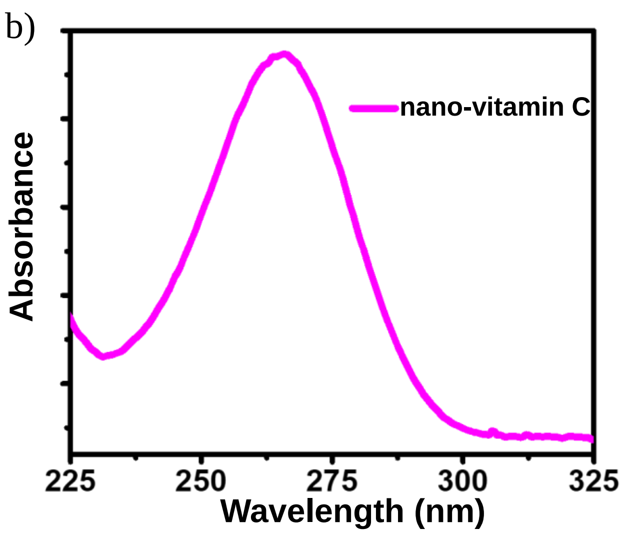


Figure S7. a) Vitamin C absorption spectra as a function of concentrations that is used to make calibration curve having equation Y=0.054X+0.111 (R^2= 0.997) where Y = Absorbance at 265 nm and X = concentration of vitamin C. This has been used for quantification of vitamin C in nanoparticle stock solution. Our results show the concentration of vitamin C in nanoparticle I stock solution as 13 mg/mL (~74 mM). b) UV visible spectra of colloidal nano-vitamin C showing the characteristic absorption band of vitamin C.

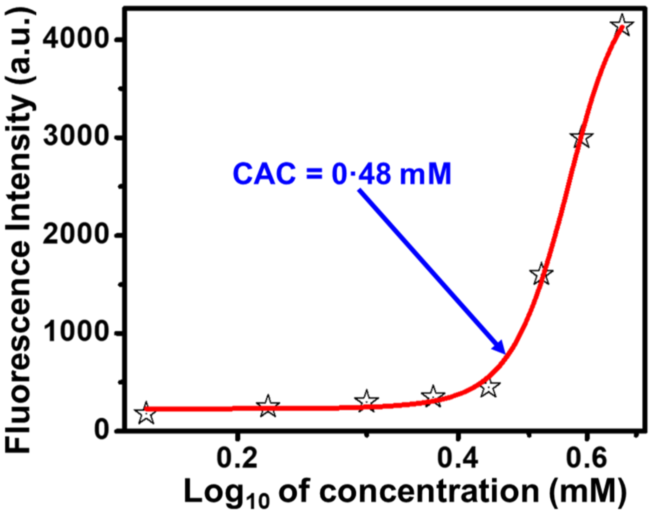


Figure S8. Determination of the critical aggregation concentration (CAC) of the compound I using nile red as a fluorescent probe by plotting log concentration of compound I against nile red fluorescence intensity. The inflection point corresponding to 0.48 mM of the compound I is as the CAC value.

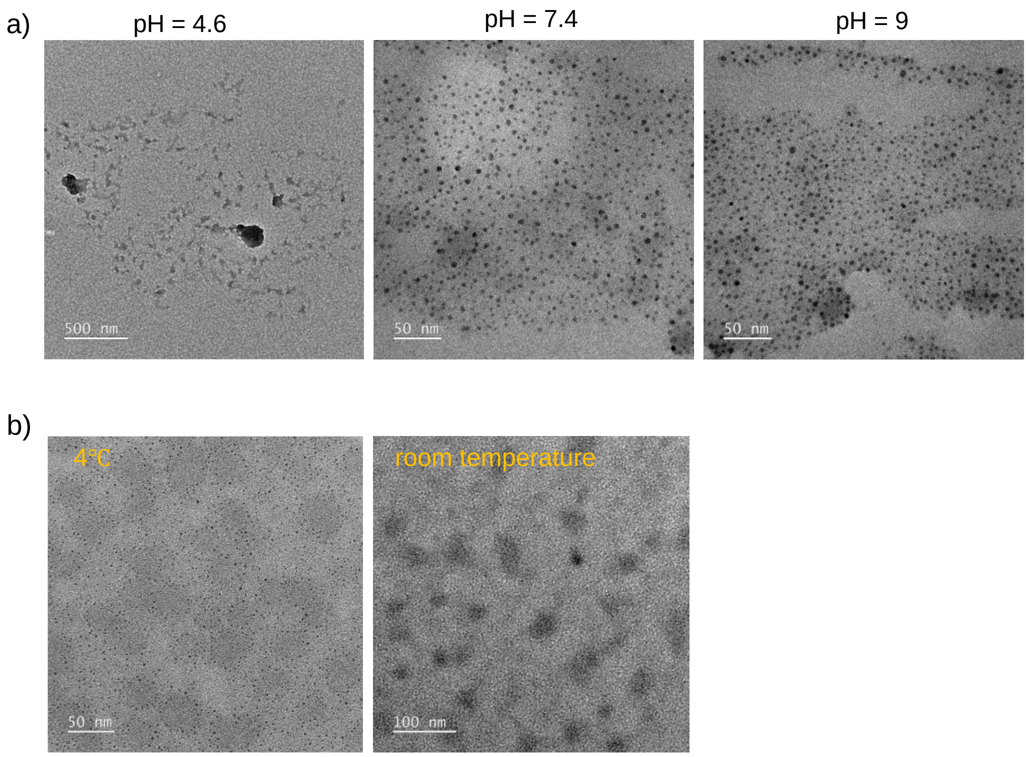


Figure S9. a) TEM image of nanoparticle I after exposing them at different pH for 24 h. b) TEM image of dispersion of nanoparticle I after keeping at 4 °C and room temperature for 7 days.

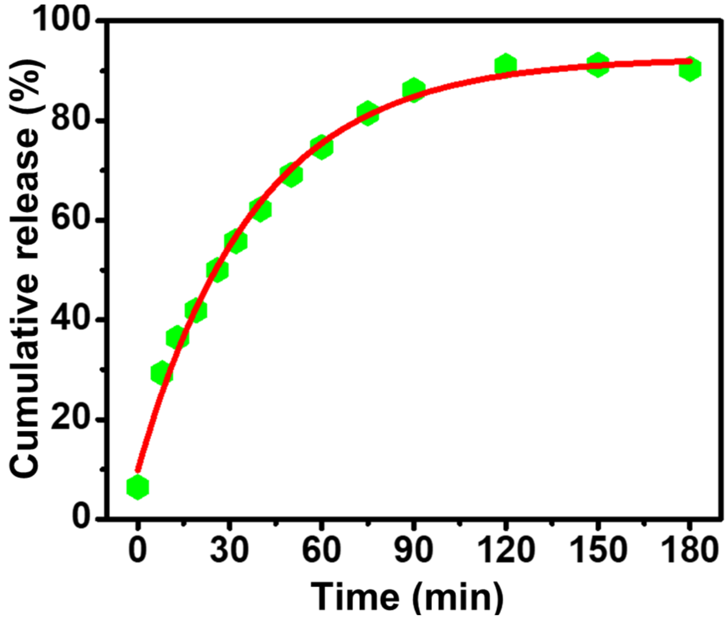
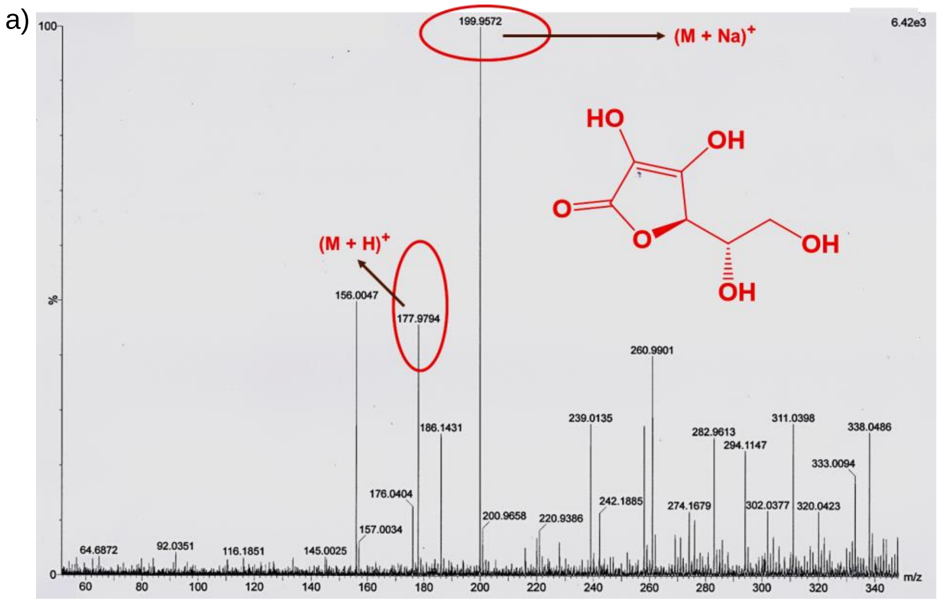


Figure S10. In vitro vitamin C release profiles from nanoparticle I at pH 4.6 buffer solution.



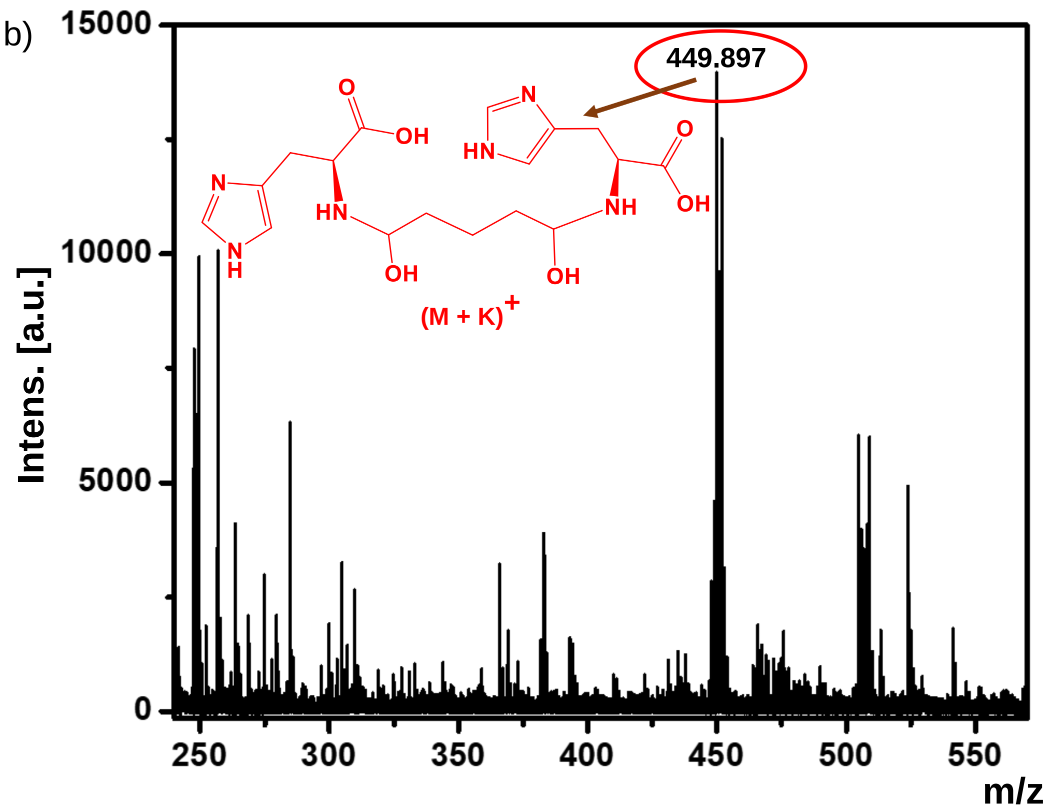


Figure S11. a) Mass spectra of the free vitamin C molecule released from nanoparticle I at pH 4.6 during dialysis against water. b) MALDI mass spectra of the remaining byproduct during the release of vitamin C molecule from nanoparticle I using dithranol as the matrix.

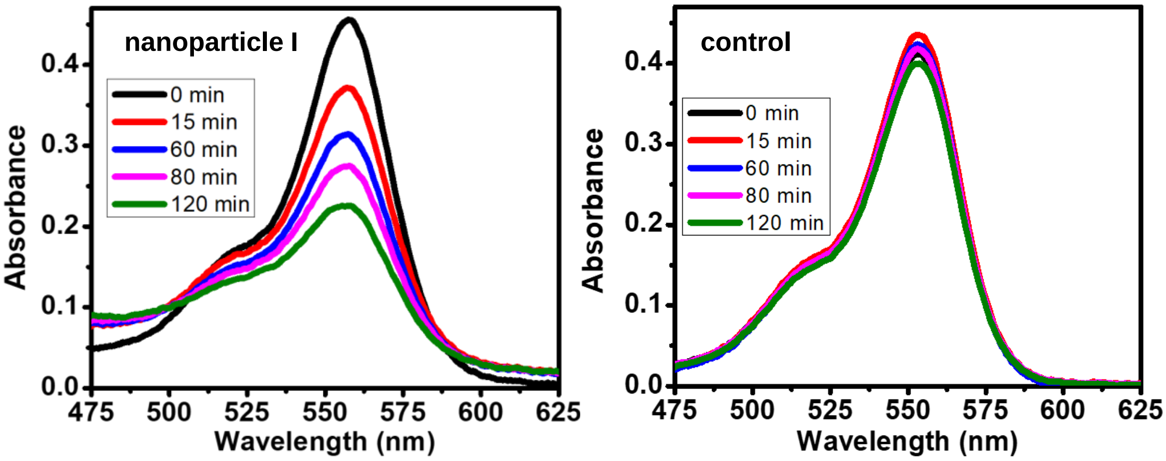


Figure S12. ROS generation by nanoparticle I evaluated using the rhodamine B dye degradation assay. A time-dependent decrease in the absorbance of rhodamine B indicates the production of reactive oxygen species (ROS). The control sample, which did not receive nanoparticle I treatment, showed no significant change, confirming the role of nanoparticle I in ROS generation.

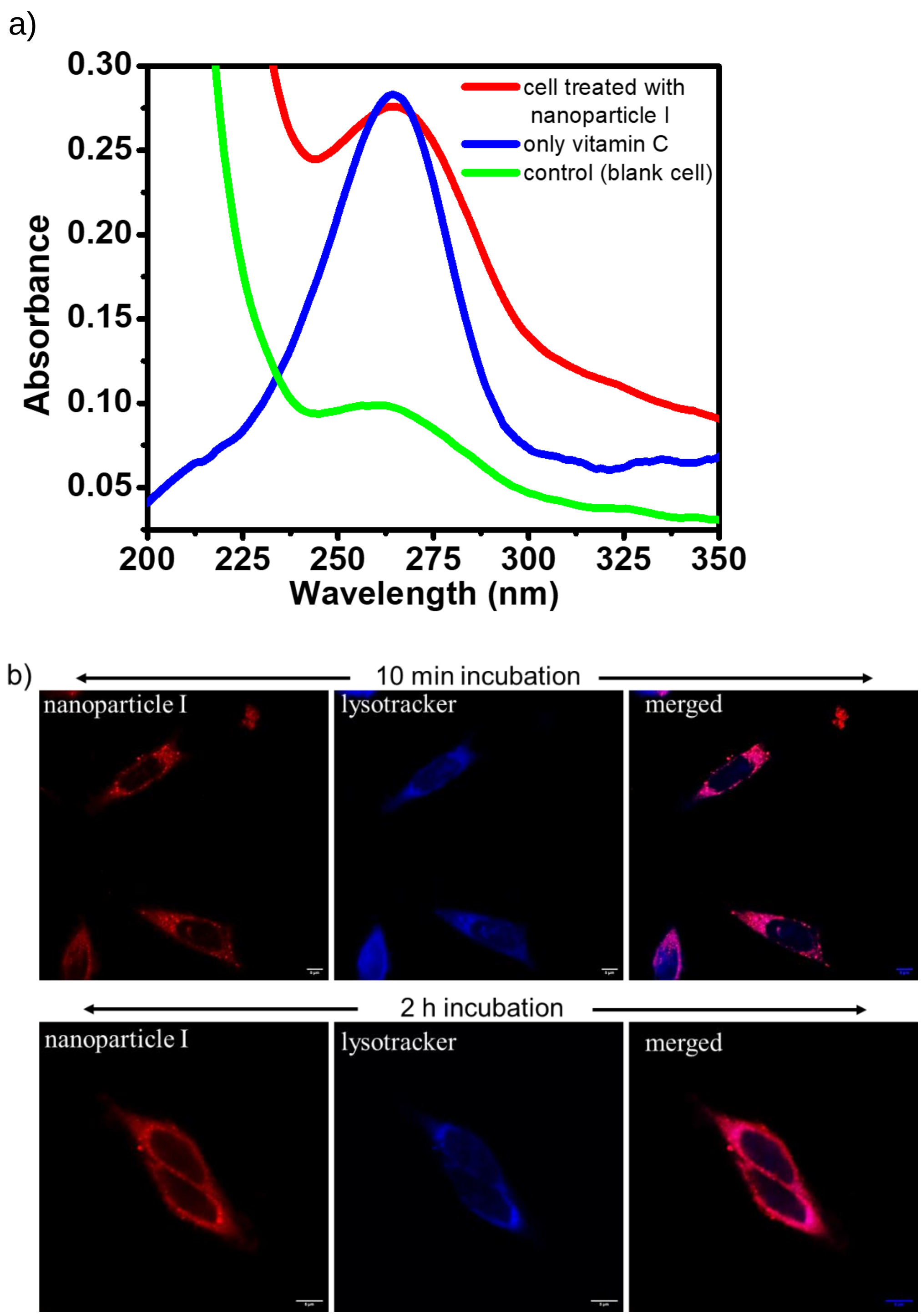


Figure S13 a) Evidence of cell delivery of the vitamin C using nanoparticle I. Typically, KB cells were incubated with nanoparticle I for 10 min. Next, washed cells were further incubated with culture media for 2h. Finally, cells were trypsinized and collected in one mL PBS buffer for UV−visible absorption spectra. Cells without treatment of any sample were considered as a control. Based on the absorbance value of vitamin C, the amount of vitamin C present in cell suspension solution was estimated to be approximately 0.3 mM. b) Subcellular localization of nile red–loaded nanoparticle I delivering vitamin C in KB cells. KB cells were pretreated with lysotracker blue for 2 hours, followed by incubation with nile red–loaded nanoparticle I (containing 2 mM vitamin C) for 10 minutes at 37 °C. The cells were then washed and further incubated in culture media for different time intervals (0 and 2 hours). Confocal microscopy images reveal that the nanoparticles predominantly colocalize with lysosomes, as indicated by the merged fluorescence signals. Scale bar represents 5 µm.

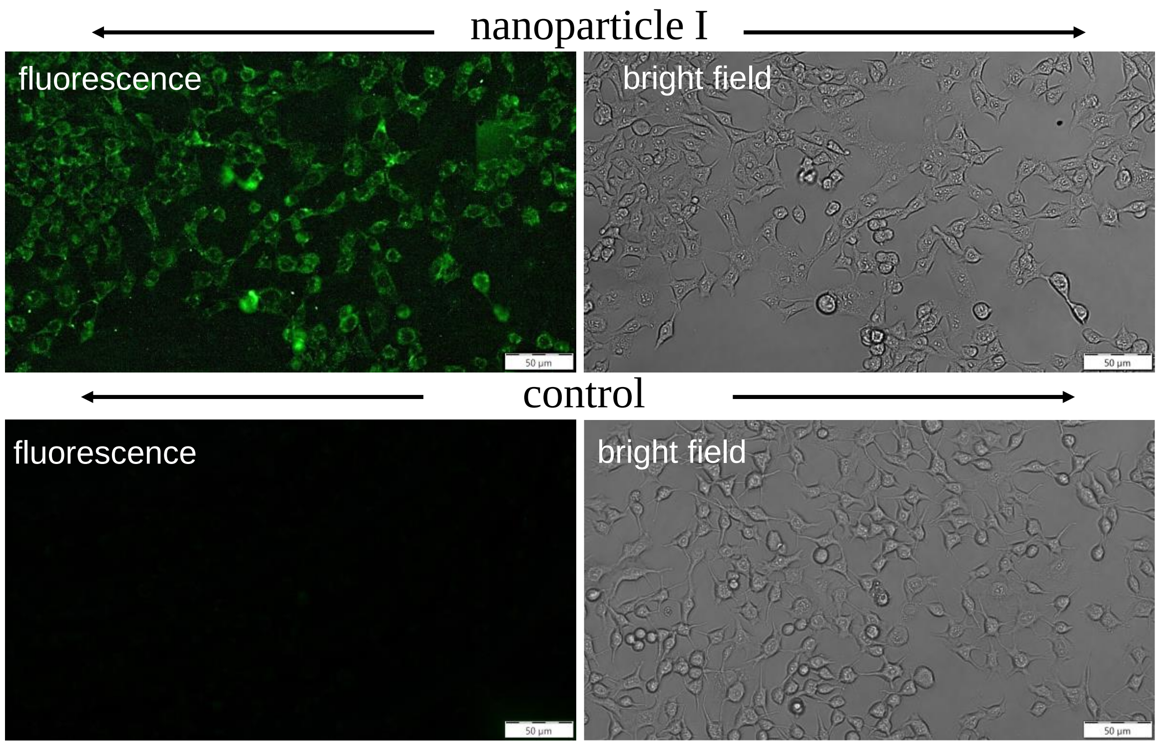


Figure S14. Intracellular generation of superoxide anion radicals in KB cells treated with nanoparticle I. Cells were incubated with nanoparticle I for 2 hours, followed by addition of 500 µL HEPES buffer containing MitoSOX (2 µM) and further incubation for 30 minutes at 37 °C. After washing, cells were imaged under a fluorescence microscope. Green fluorescence indicates the presence of superoxide radicals. Untreated cells served as the control. Scale bar: 50 µm.

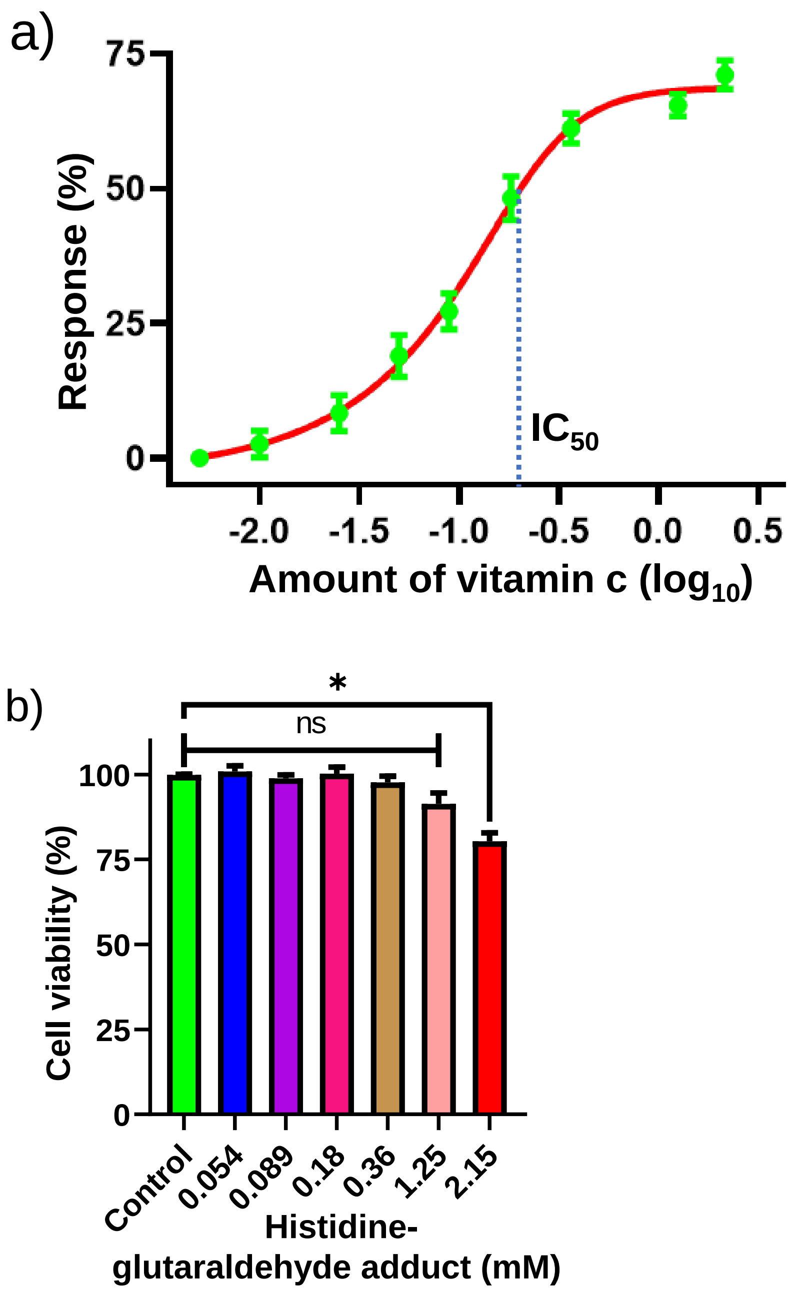


Figure S15. a) Dose–response curve of nanoparticle I plotted with varying vitamin C concentrations (0.01–2 mM) for precise IC_{50} value determination. The curve was fitted using Richard's five-parameter dose–response equation in GraphPad Prism 8.0.2. The obtained Hill slope value of 2.23 indicating a strong threshold effect in the cellular response. b) MTT based cytotoxicity assay of histidine-glutaraldehyde (carbinolamine byproduct) at five different doses after 24 h incubation. Cell viability was investigated by assuming 100 % cell viability for the cells with no sample treatment (control). The data represented the mean of N = 3 experiments (*$p < 0.05$, ns = non-significant) via Welch's ANOVA with Brown-Forsythe test.

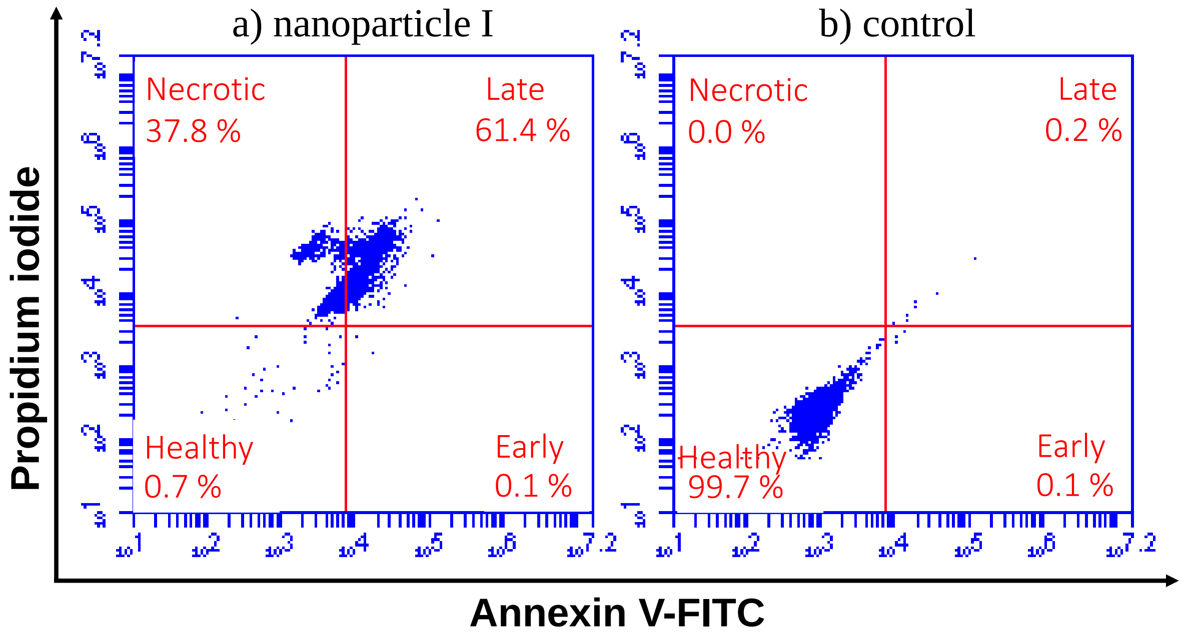


Figure S16. Assessment of cellular apoptosis/necrosis level after treatments with nanoparticle I (vitamin C is 0.58 mM) a). KB cells are treated with nanoparticle I for 24 h followed by FACS analysis via annexin V-FITC and propidium iodide staining. b). Control refers to cell without treatment of any sample.