Supporting Information

Designing a Nanothermometer using Gel-to-Liquid Phase Transition Property of Hybrid Niosome

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S1. Experimental section

Materials and methods

S1a. Materials

Span 60, Pluronic L-64, Cholesterol with purity ≥ 99%, and Coumarin 153 were purchased from Sigma Aldrich. Methanol (HPLC grade) and chloroform (HPLC grade) with a purity of 99% were purchased from Finar, India.

S1b. Preparation of niosome S60-L64 (80:20) hybrid niosomes

Niosomes were prepared by a thin-layer evaporation method. Span60 (S60) and L64 (PEO_{13}-PPO_{30}-PEO_{13}) a triblock copolymer composed of hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) (PPO) polymer units were used as non-ionic surfactants for synthesizing hybrid niosomes. Firstly 5 mM of Cholesterol was taken and added to S60-L64 taken in an 80:20 millimolar ratio (8 mM Span 60 and 2 mM L64) and dissolved in a (2:1) mixture of chloroform and methanol in a round bottom flask. A rotary evaporator was used to evaporate the solvent, a vacuum of 20 Hg at 30°C and 100 rpm conditions were maintained until a thin film was formed in the round bottom flask, and hydration of the formed film was done using 6 mL of Millipore water resulting in a colloidal solution. The colloidal suspension was vortexed for 5 minutes followed by sonication for 30 minutes to form the hybrid niosome stock solution. 1 mL of this stock niosome solution was diluted 50 times with Millipore water to get the working standard of hybrid niosomes.

S1c. Preparation of Coumarin 153 loaded S60-L64 hybrid niosomes

A stock solution of coumarin 153 of 1mM was prepared in pyridine, then an appropriate amount of the stock solution of C153 was added to the working standard of niosomes to get the final concentration of C153 to 10^{-5} M. Incubation time of 3 hours was given after adding the dye to form C153 10^{-5} M ex-situ loaded S60-L64 hybrid niosomes.

S1d. Characterization

Niosomes were characterized by using dynamic light scattering (DLS) and Field Emission Scanning Electron Microscope (FE-SEM) techniques. For SEM sample visualization, the stock solution of niosome was diluted with water to a transparent solution and the diluted niosomal suspension was then drop-casted on a clean silicon wafer and kept for drying. The

size and morphology of the niosome could be visualized through SEM. The same procedure was followed for STEM imaging; however, Cu grids were used instead of Si wafer. Malvern particle size analyzer (zeta sizer nano-ZS) was used to determine the hydrodynamic radius and the zeta potential of the niosomes.

S1e. Fluorescence Measurements

The temperature-dependent fluorescence measurements for the C153 1×10^{-5} M ex-situ loaded S60-L64 hybrid niosomes were performed on the Horiba Fluorolog spectrofluorometer. The emission spectra of the niosomes were recorded by fixing the excitation wavelength at 420 nm, between 20°C and 50°C. The excitation and emission slit widths were kept at 1.4 nm, during the measurement. The samples for fluorescence measurements were prepared by taking 1980 µL of working standard of niosomes and 20 µL of C153 (1 mM stock in pyridine) was added and kept for incubation for 3 hours to get C153 (10^{-5} M) ex-situ loaded S60-L64 hybrid niosomes. 2mL of this dye loaded niosome solution was placed in a quartz cuvette and fluorescence measurements were taken at different temperatures.

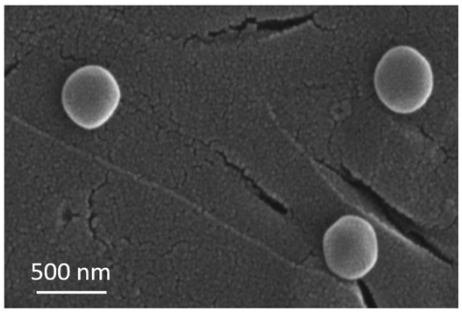

Figure S1: FE-SEM images of S60-L64 hybrid niosomes.

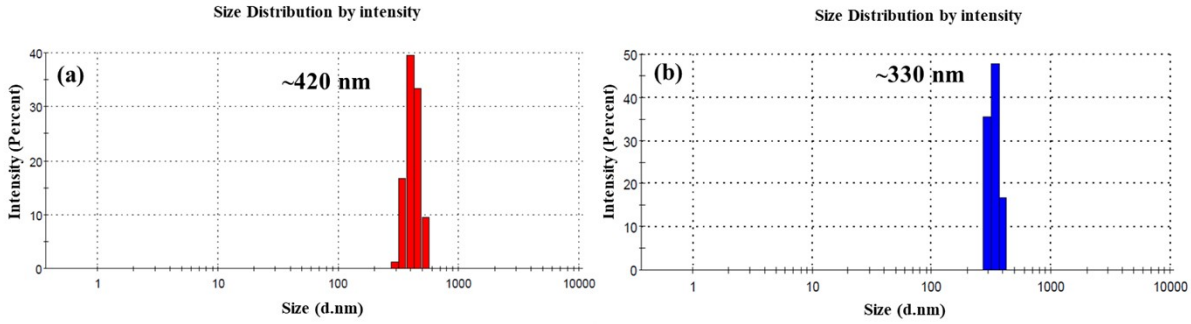

Figure S2: DLS data of S60-L64 hybrid niosomes (a) before extrusion filtration and (b) after extrusion filtration

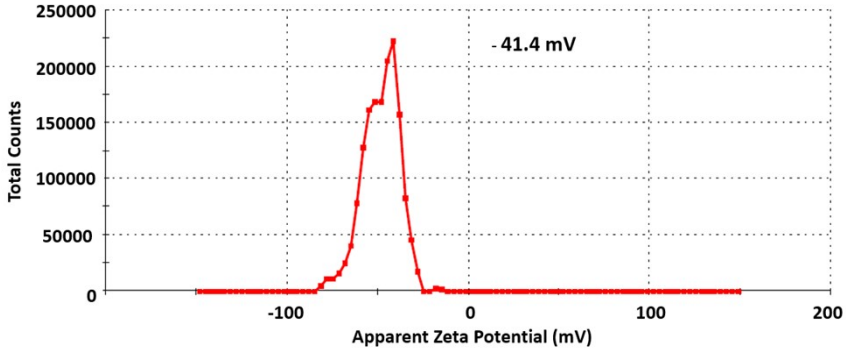

Figure S3: Zeta potential of S60-L64 hybrid niosomes.

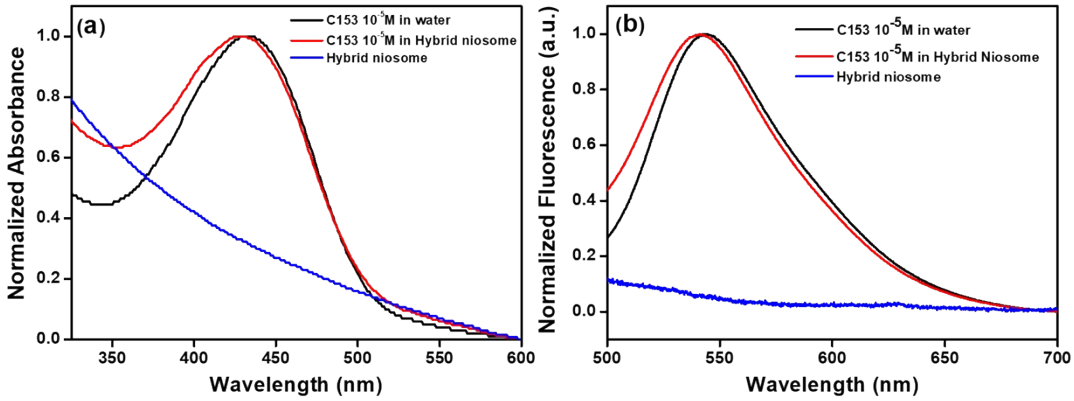

Figure S4: (a) Normalized Absorbance spectra and (b) Normalized fluorescence spectra of C153 (10^{-5}M) in water, C153-loaded S60-L64 hybrid niosomes and only hybrid niosome.

S2: $E_T(30)$ study of C153 to confirm the location in hybrid niosome

$E_T(30)$ study was performed to determine the location of the probe C153 in the niosome environment. First, solutions of different percentages of Dioxane-water mixture were prepared, and then the same amount of fluorophore in each of the solutions was dissolved. The standard E_T30 parameters are known from the literature for each of the Dioxane-water mixtures with varying percentages (see the below **Table 1**). Next, emission spectra of each of the mixture containing the fluorophores were recorded, and a correlation plot was obtained (figure below) from the emission maxima of both fluorophores in a different composition of dioxane water mixture against the solvent polarity parameter ($E_T(30)$). There is a shift in the fluorescence emission for C153 from 545nm to 516nm from hydrophilic to hydrophobic environment. Then the sample fluorophore was entrapped in hybrid niosome solution and its fluorescence maximum was recorded and compared with the calibration curve values obtained already. Accordingly, the location of the fluorophore in the niosome environment was confirmed. For C153 in the hybrid niosome, the $E_T(30)$ value was found to be 57.2 Kcal/mol, thus indicating that C153 probably resided near the interface of the hydrophilic region of the niosome.

Table S1. $E_T(30)$ values of different dioxane-water compositions.

Water (mL)	Dioxane (mL)	E_T (30) Values	$\lambda_{emission}$ of C153
5	0	61.8	545.2
4.5	0.5	60.9	541.2
4	1	58.2	541.0
3.5	1.5	57.2	539.8
3	2	55.8	537.2
2.5	2.5	53.2	535.2
2	3	52	533.0
1.5	3.5	50.5	530.2
1	4	48.7	527.4
0.5	4.5	46.6	523.6
0	5	36.3	516.6

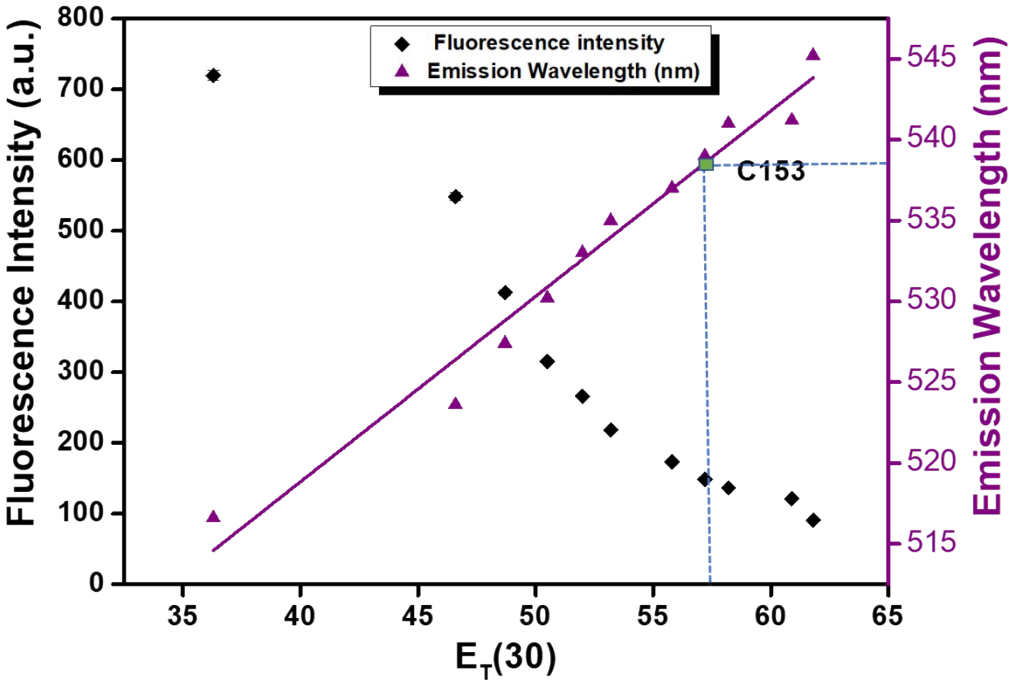

Figure S5. Estimation of the $E_T(30)$ value of C153 in hybrid niosome

From the temperature-dependent fluorescence studies of C153 (10^{-5} M) niosomes, it is observed that there is a blue shift of emission with an enhancement in intensity as we increase the temperature from 20°C to 50°C. It suggests that C153 moves from the hydrophilic region of the niosome to the hydrophobic bilayer of the niosome with an increase in temperature.

Table S2: Comparison of the relative sensitivity of various temperature sensors.

Nano-Thermometer material	Temperature range	λ_{ex} (nm)	λ_{em} (nm)	S_r^{max} (T)	Application	Ref.
Lanthanide-ion-doped nanoparticles LaF_3:Nd	32−53°C (305-326 K)	808	880	0.25% K^{-1} (33 °C)	Photothermal therapy (PTT) with temperature feedback	1
PbS/CdS/ZnS	30−67°C (303-340 K)	808	1,220	1% K^{-1} (20 °C)	PTT therapy with temperature feedback	2
Ag_2S	$\Delta T \leq 7$°C (280 K)	808	1,200	3% K^{-1} (28 °C)	Tumour diagnosis	3
Formamide, glutathione Based carbon dots	5-60°C	640	645-730	3.71% $°C^{-1}$	For in vitro and possibly in vivo fluorescence nano thermometry	4
Nitrogen and boron co-doped CDs	0-90°C	340	450	1.8%$°C^{-1}$	Nanothermometer and Fe^{3+}/Fe^{2+} sensor	5
$NaGdF_4$: Nd^{3+}	298–323 K	808	1060/1220	2.5×10^{-2} $\%K^{-1}$ at 30°C	High-Sensitivity Luminescence Nanothermometry in the Second Biological Window	6
Rhodamine Dye-Incorporated F127-Melamine-Formaldehyde Polymer Nanoparticle	20-90°C	488	510-650	15.4%$°C^{-1}$	Monitoring cellular temperature variations	7
MPB-PEG4000	-50−100°C (223-373 K)	335	430/530	0.14% K^{-1} at 10°C (283 K^{-1})	Luminescent thermometer in a solid-state polymer	8
Dye-conjugated upconverting nanoparticles	20−50°C (293-323 K)	980	550/670	0.9% K^{-1}	To detect the temperature at a subcellular level in the physiological range	9
Red-Emitting Carbon nanodots (RCDs)	4−80°C (277-353 K)	500	emission peaks are mainly centered at ~650 nm	~1.2% K^{-1}	Nanothermometer for intracellular temperature sensing	10
PbS/CdS/CdSe QDs	-93−27°C (180-300 K)	560	670/910	1.22%K^{-1} attained at 27°C	Potential of visible and NIR luminescence	11

				(300 K)	nanothermometers based on dual-emission PbS/CdS/CdSe QDs for several technologies	
C153 loaded (80:20) S60-L64 hybrid niosome	38−50°C (311-323 K)	420	540-520	7.4% $°C^{-1}$ ($7.4\%K^{-1}$) at 50°C (323 K)	For intracellular temperature sensing	Current work

S3. Temperature uncertainty calculation of C153-loaded S60-L64 hybrid niosomes at 50°C

Temperature uncertainty δT is the smallest change in temperature that causes a perceptible change in the indication. Mathematically it is given by the following equation

$$\delta T = \frac{1}{S_r} \times \frac{\delta FIR}{FIR}$$

Where Sr is the relative sensitivity, δFIR is the standard deviation of fluorescence intensity of 15 readings at 50°C and FIR represents the mean fluorescence intensity of 10^{-5} M C135 loaded S60-L64 Hybrid niosomes of 15 readings. The temperature sensitivity was found to be 0.087°C at 50°C.

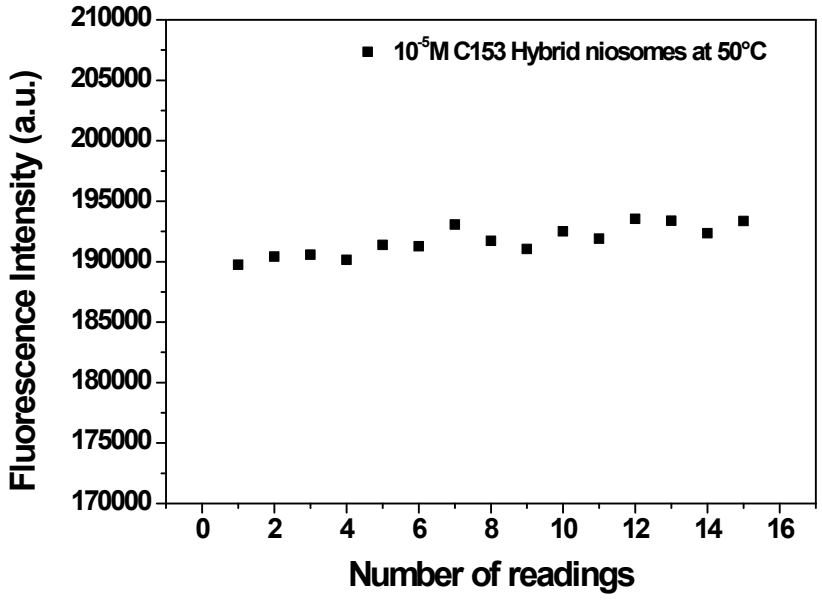

Figure S6: Fluorescence Intensity (collected at 520 nm) readings of C153-loaded S60-L64 hybrid niosomes taken at 1-minute interval till 15 minutes, at 50°C.

S4. Confocal imaging

The samples for confocal studies were prepared by diluting the niosomes stock with Millipore water 50 times and C153 was loaded into them in an ex-situ manner such that the final concentration of C153 inside the niosome was 10^{-5} M. 10µl of niosome drop was placed on a glass slide and covered with a coverslip and sealed for 1 hour before confocal imaging. The excitation laser source of 405 nm with a laser power of 4% (9.36 µW) was used. A stage top incubator from OKO Labs was used to vary the temperature from 25°C to 50°C. The coverslip was placed in the incubator and the images were taken after 5 minutes of incubation time after every successive temperature. The emission window for C153 loaded niosomes was 510 nm to 530 nm, since the increase in fluorescence intensity at higher temperatures is seen in this range.

For cellular confocal imaging, FaDu cells were seeded on a cover slip and placed in a 12-well plate (5 x 10^4 cells per well) and incubated for 24 h at 37°C. Confocal laser scanning microscopy was performed to visualize the localization of niosomes within cells and understand the change in fluorescence intensity with the increase in temperature. Based on the results from cell viability studies, 10^{-5} M concentration was used for bioimaging studies. Cells were treated with C153 niosomes for 4 h. Then, the cells were washed twice with phosphate-buffered saline. Cells were fixed by incubating in 4% paraformaldehyde for 10 minutes and then washed twice with PBS. The cells on the coverslip were mounted on a microscope slide using a Flouroshield® mounting medium. The cells were visualized using a confocal laser scanning microscope (TCS SP8, Lecia Microsystems, Germany). C153 was excited at a wavelength of 405 nm and the emission was captured at a range of 510-530 nm. The change in fluorescence intensity of C153 at 25-50°C was captured at the magnification of 20X. The temperature was maintained using a temperature controller (H301-EC-M, OKO Labs, USA) with a temperature accuracy of 0.1^0C.

S4a. Fluorescence intensity calculation from the confocal images

Confocal images of C153 loaded niosomes and C153 loaded niosomes in cells were taken using a Leica confocal microscope. The fluorescence of the C153 loaded niosomes was measured by selecting an area of interest and the fluorescence intensity was obtained from the mean value of the selected area provided by the Leica software as shown in figures (S7, S8, S9, and S10) similarly the fluorescence intensity of C153 loaded niosomes was determined by selecting a region of interest over the selected area of cells and the mean value was

obtained from leica software which gives the fluorescence intensity of that particular area. The same procedure was followed to measure the fluorescence intensity at all temperatures.

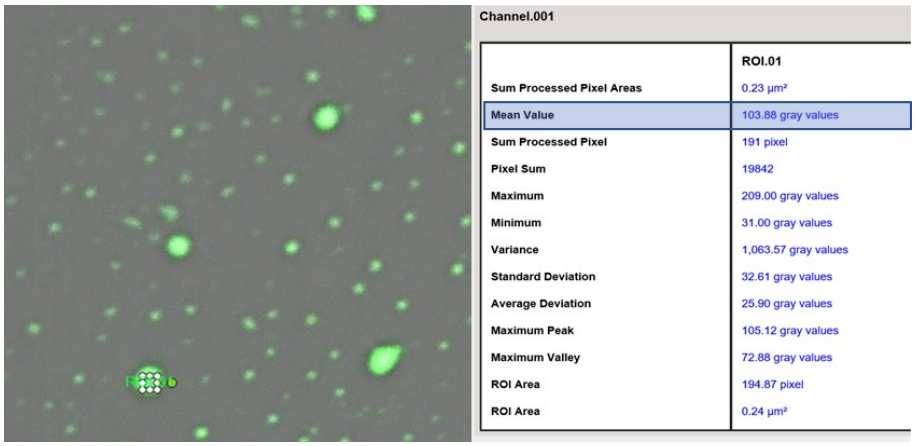

Figure S7: Confocal image and corresponding fluorescence intensity of C153 (10^{-5} M) loaded S60-L64 hybrid niosomes at 25 °C.

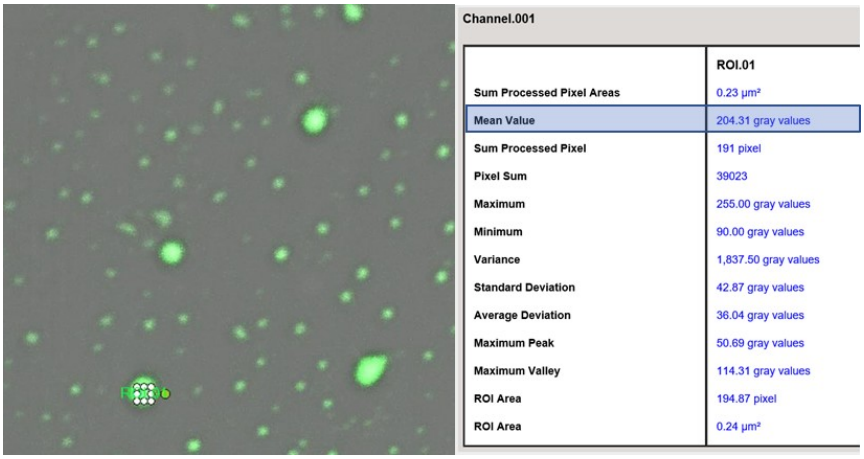

Figure S8: Confocal image and corresponding fluorescence intensity of C153 (10^{-5} M) loaded S60-L64 hybrid niosomes at 50°C.

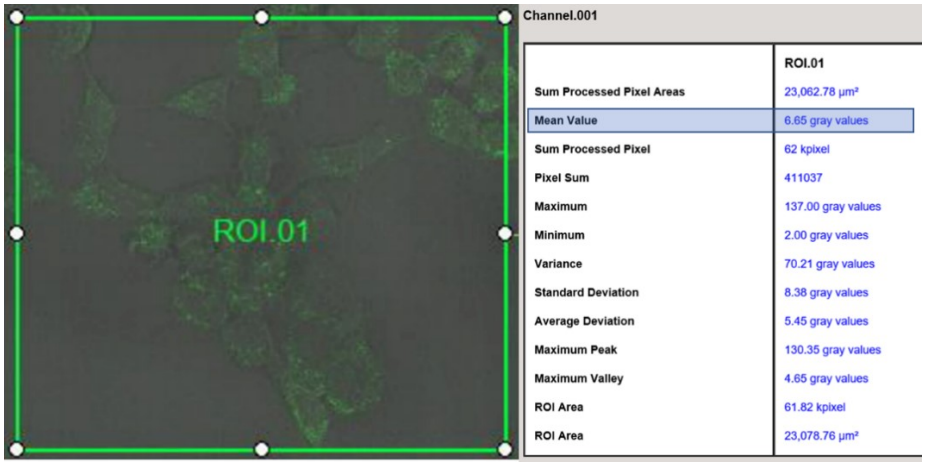

Figure S9: Confocal image and corresponding fluorescence intensity of C153 (10^{-5} M) loaded S60-L64 hybrid niosomes in FaDu cells at 25 °C.

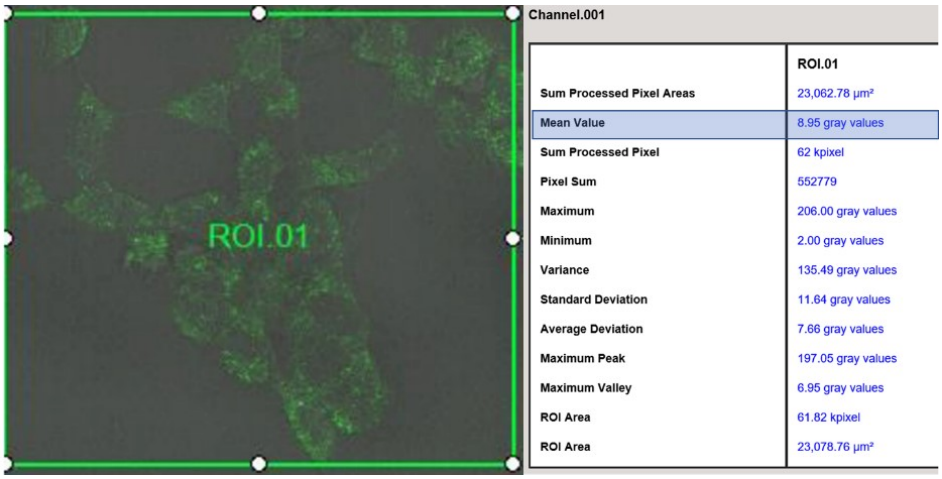

Figure S10: Confocal image and corresponding fluorescence intensity of C153 (10^{-5} M) loaded S60-L64 hybrid niosomes in FaDu cells at 50 °C.

S5. Reproducibility of fluorescence data of C153 (10^{-5} M)-loaded S60-L64 hybrid niosomes using different instruments

The reproducibility of experimental results is a crucial aspect of scientific research, as it enables the validation of findings and ensures the reliability of the data. The ability to reproduce results under different experimental conditions and with different instruments is particularly significant as it enhances the robustness and generalizability of the findings. A comprehensive evaluation of fluorescence enhancement of C153 (10^{-5} M)-loaded S60-L64

hybrid niosomes with temperature was done using a Hitachi-7000 fluorescence spectrophotometer and Horiba Fluorolog. From (figure S11 (a) and (b)) we observe an enhancement in fluorescence intensity with increasing temperature, which was consistent across both instruments.

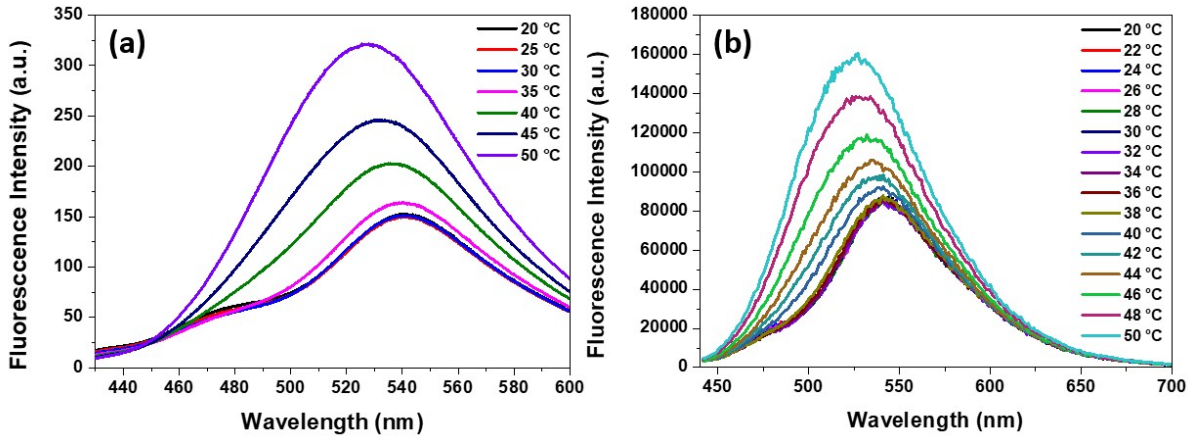

Figure S11: Reproducibility data of fluorescence intensity of C153 (10^{-5} M) ex-situ loaded S60-L64 hybrid niosomes in (a) Hitachi-7000 fluorescence spectrophotometer and (b) Horiba Fluorolog at different temperatures.

S6: CIE plot of C153 (10^{-5} M) ex-situ loaded S60-L64 hybrid niosomes

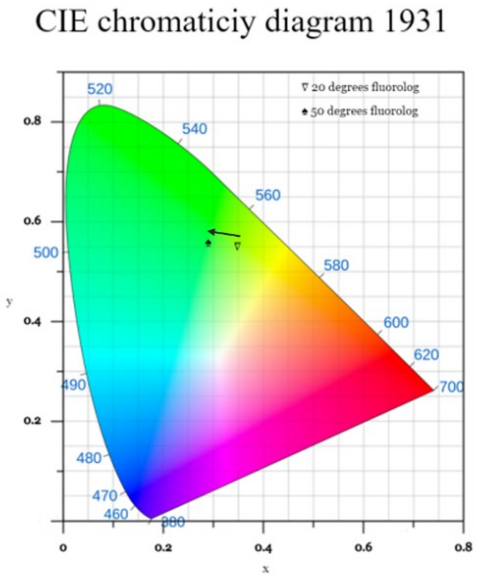

Figure S12: CIE plot of C153 (10^{-5} M) ex-situ loaded S60-L64 hybrid niosomes in Hitachi-7000 fluorescence spectrophotometer at 25 °C and 50 °C different temperatures.

Based on the commission international d'Eclairage (CIE) chromaticity diagram, the chromaticity coordinates of the fluorescence spectra (Figure S12) of C153 (10^{-5} M) loaded niosomes dependent on temperature and changed from (0.348,0.55) at 20 °C to (0.29,0.558) at 50 °C which was consistent with the color change observed by the naked eye or in camera.

S7. Determining the suitable concentration of C153 dye for temperature sensing

Standardization of coumarin concentration to be loaded in niosomes for temperature sensing was done by loading different concentrations of coumarin in hybrid niosomes and studying their fluorescence intensity with an increase in temperature. The concentrations of C153 in niosomes that were taken were (a)1×10^{-5}M, (b) 2.5×10^{-5}M, (c) 5×10^{-5}M, and (d) 1×10^{-4}M. From (figure S13) we can see that the fluorescence increase is maximum in C153 (10^{-5} M) hybrid niosomes and the least increase can be seen in C153 (10^{-4} M) hybrid niosomes. Hence C153 (10^{-5} M) loaded hybrid niosomes were used as the optimum concentration for temperature sensing application.

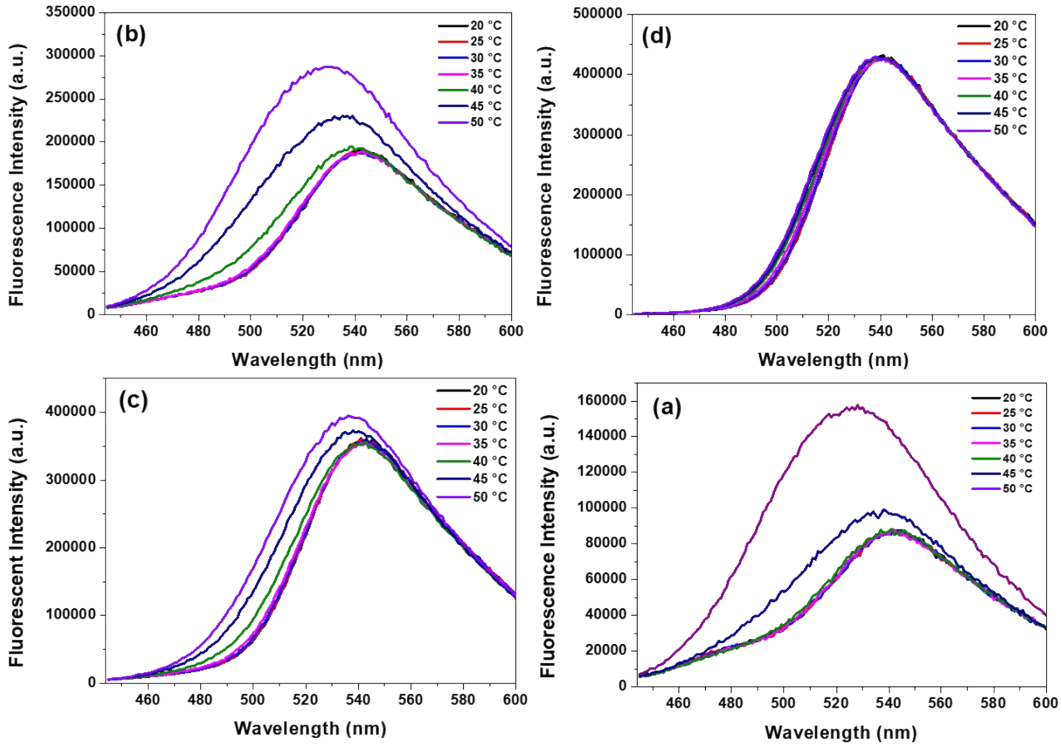

Figure S13: Different concentrations of C153 dye loaded in S60-L64 hybrid niosomes and their fluorescence change with temperature.

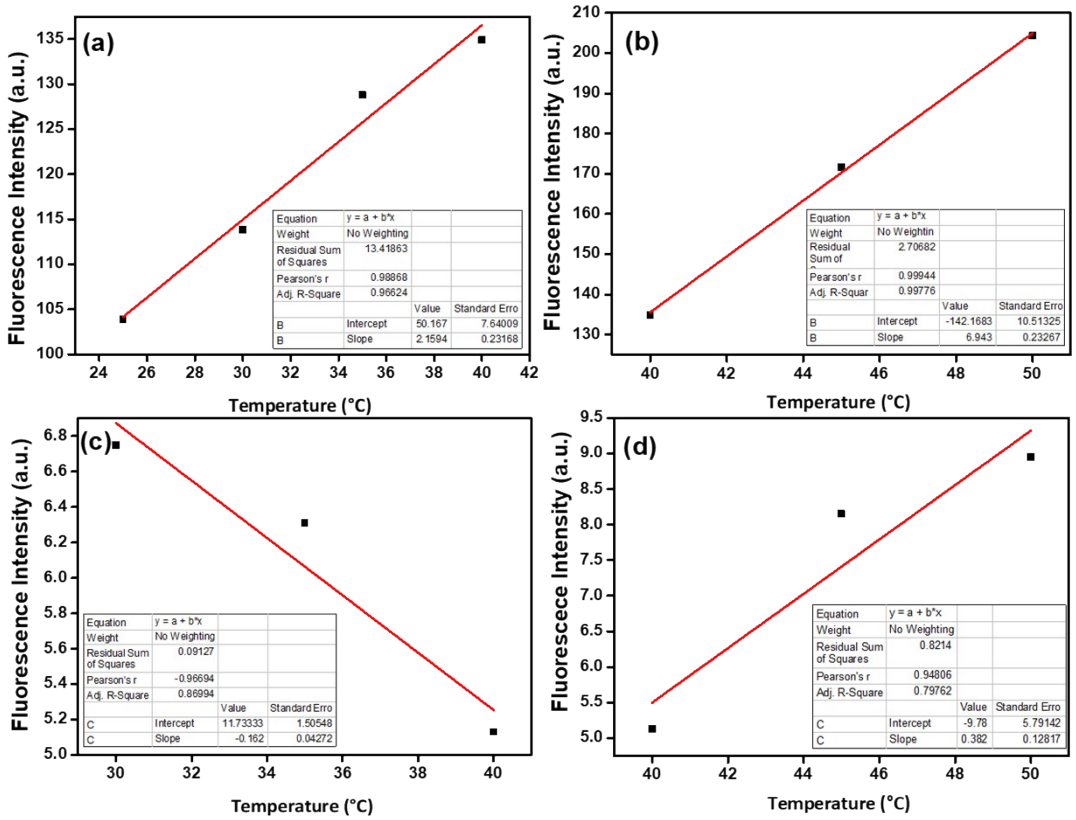

Figure S14: Slope calculation of the fluorescence intensities obtained from confocal images as a function of temperature in two temperature ranges: (a, b) C153 in S60-L64 hybrid niosomes and (c, d) C153 S60-L64 hybrid niosomes in FaDu cells.

S8. Cell viability studies

FaDu cells (human oropharyngeal carcinoma cells) were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), and 1% penicillin. Cells were incubated at 37±0.2 ºC in 5% CO_2 and 95% relative humidity in an incubator.

MTT assay was performed to study the cell viability after incubation with different concentrations of C153 niosomes. Cells were seeded in a 96-well plate (1 x 10^5 cells per well), and incubated for 24 h at 37 ℃. Niosomes were resuspended with RPMI medium containing 2% FBS. Cells were treated with blank niosomes, C153 niosomes, and neat C153 solution at 0.75 x 10^{-5} to 1.25 x 10^{-5} M concentration for 4 h. Untreated cells acted as a control group. Then, MTT solution (5 mg/ml) was added to each well and incubated for an additional 4 h. Later, 100 µl of DMSO was added to each well to dissolve the formazan crystals. Absorbance was recorded using a microplate reader (Spectra Max M4) at 570 nm, and % cell viability was calculated using equation 1.

$$\% \, Cell \, viability = \frac{O.D \, of \, control \, cells - O.D \, of \, treated \, cells}{O.D \, of \, control \, cells} X \, 100 \quad (1)$$

Where O.D. stands for the optical density of the respective solutions at 570 nm. The resulting data were reported as mean ± standard deviation values. Two-way ANOVA followed by the Tukey test was used to determine the statistical difference between different groups.

S9. Cell uptake studies

The cell uptake of C153 niosomes was studied by flow cytometric analysis (BD FACS Aria III, USA). FaDu cells were seeded in a 6-well plate (5 x10^4 cells per well) and for 24 h at 37 °C. C153 niosomes were incubated with cells for 4 h. Untreated cells were kept as control. Then, cells were washed twice with PBS, trypsinized, and resuspended with ice-cold PBS. The cell uptake was determined by shift of histogram area in the FITC channel in terms of fluorescence intensity at 135, 280, and 330 volts for FSC, SSC, and FITC respectively.

S10. Calculation of maximum and minimum repeatability at 50 °C

The maximum and minimum repetition rates can be calculated by using the repeatability studies of temperature-dependent fluorescence of 10^{-5} M C153 Hybrid niosome at 20 °C and 50 °C. The repeatability (R) is given by the formula.

$$R = 1 - (\frac{FI_{mean} - FI_{actual}}{FI_{mean}})$$

Where the FI mean is the mean value of six fluorescence readings at 50 °C and FI actual represents the value of fluorescence intensity showing maximum and minimum deviations from the mean.

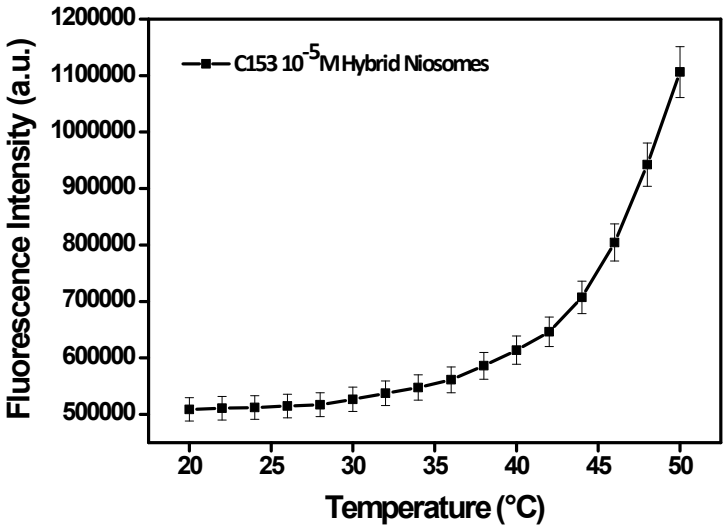

Figure S15. The variation of fluorescence intensity (collected at λ_{max} at each temperature) of C153 (10^{-5}M)-loaded Hybrid niosomes with temperature.

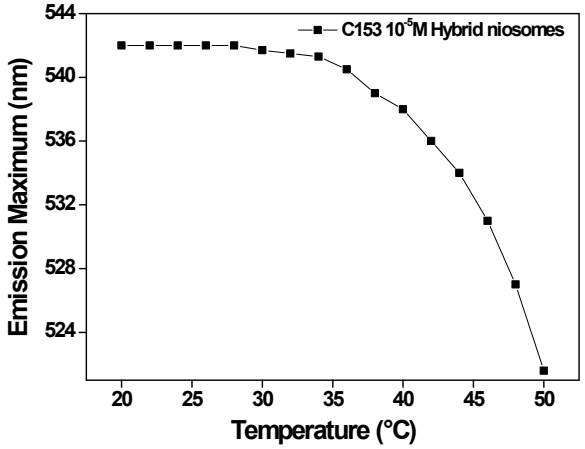

Figure S16. The variation in fluorescence emission maximum of C153 (10^{-5}M)-loaded Hybrid niosomes with temperature.

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