Electronic Supplementary Material (ESI) for Chemical Science.

Supporting Information

Destructive Spontaneous Ingression of Tunable Silica Nanosheets through Cancer Cell Membrane

Arghya Bandyopadhyay,[1] Priya Yadav,[2] Keka Sarkar,[2] and Sayan Bhattacharyya*,[1]

[1]*Department of Chemical Sciences, and Centre for Advanced Functional Materials, Indian Institute of Science Education and Research (IISER) Kolkata, Mohanpur - 741246, India*
[2]*Department of Microbiology, University of Kalyani, Nadia – 741235, India*

*Email for correspondence: sayanb@iiserkol.ac.in
Tel: +91-33-6136-0000 Ext. 1275

Discussion S1: Materials and Methods

For the synthesis of silica nanosheets (Si-NSs), methanol and ammonium hydroxide solution were purchased from Merck, India and used without further purification. The primary antibody Anti-ST6GALNAC1 (ab69066) and the secondary antibody Rabbit anti-mouse IgG H&L Alexa Fluor® 488 (ab150125) were purchased from Abcam Inc, Toronto, ON M5W 0E9. All other chemicals used were purchased from Sigma-Aldrich and directly incorporated in the experiments. In all experiments deionised nanopure water (filtered through a 0.015 μm membrane filter, Nuclepore, Whatman) was used and then autoclaved, if required, for 30 min at 121°C. Zeta potential was measured by a Malvern Zetasizer Nano ZS dynamic light scattering instrument. AFM analysis was performed by Veeco di CP-II AFM instrument, wherein the as-prepared methanolic dispersion of NSs was drop-casted on cleaned and dried silicon wafers. TEM and HAADF-STEM images with EDS mapping were recorded using JEOL 2100F microscope at an accelerating voltage of 200 kV. For TEM imaging the samples were drop casted on carbon coated copper grids (mesh size 300, TED Pella, USA) and dried under vacuum. The XRD measurements were carried out with a Rigaku (mini flex II, Japan) powder X-ray diffractometer having Cu Kα = 1.54059 Å radiation. Zeiss Supra 55 VP FESEM was used for recording the three-dimensional (3D) images of the treated cells. For FTIR spectral analysis, PerkinElmer spectrum RX1 spectrum equipped with liquid sample analyzer was used. Fluorescence microscopic characterization of cultured animal cells was done with Olympus, IX51 attached to JENOPTIK® ProgRes® MF cool CCD camera. Flow cytometric data were obtained by BD FACS-Versa™, (Becton-Dickinson, BD Biosciences) with Cell Quest Pro software and re-analyzed using FlowJo. ICP-MS measurements were carried out in a Thermo

Scientific Xseries-2 with Plasma lab software. Other relevant and specific information is provided in the individual sections.

Synthesis of Silica NSs

Thinnest Unmodified NSs: For the synthesis of NSs, the following formulation was standardized. In a 25 mL HPLC grade methanolic solution of NH_4OH having pH 8.5, 50 μL of 100 mM methanolic CTAB solution was added and mixed thoroughly. In the above solution, 25 mL 0.01 M methanolic TEOS solution was mixed and ripened under microwave irradiation for 5 min, at 60°C and 900 W. During the ripening of the mixture the solution was kept under reflux condition with continuous stirring of 500 rpm with a magnetic stirrer. This sample is named U1.

Thicker Unmodified NSs: The as-prepared colloidal NS solution was kept under ambient conditions to allow spontaneous aggregation through NS stacking. The samples were collected after 3, 5 and 7 days and named as U3, U5 and U7, respectively.

Surface Modification of the NSs

Modification with DOX and/or folic acid: The NS surface was modified with DOX and folic acid separately, along with a co-modification with both DOX and folic acid. A methanolic solution of DOX and/or folic acid was added to 2 mL of NS suspension to give a final concentration of 100 ng/mL. The mixture was then kept under continuous stirring at 1500 rpm overnight at 4°C to facilitate the loading of the drugs onto NSs. The loaded NSs were separated by applying 65,000 relative centrifugal force (rcf) and the obtained pellet was re-suspended in 1 mL 0.2 mM phosphate buffer saline (PBS) (pH 7.4) to obtain the D, F and DF series of NSs.

Modification with antibody: For exclusively targeting the HeLa cancer cells, the primary antibody was first conjugated on the surface of U1 NSs. 1:500 dilution of the primary antibody was incubated with the NS suspension in PBS (pH 6.0) under stirring overnight at 4°C. After conjugation, the modified NSs were washed with PBS by centrifugation and dispersed in PBS before treatment on different cell types.

Cell Culture

The human cervix adenocarcinoma (HeLa) cells and Human embryonic kidney cells (HEK-293) (NCCS, Pune, India) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin G, and 100 µg/mL streptomycin in a humidified incubator with 5% CO_2 in the humidified air at 37°C. After reaching the confluency, the cells were detached from the flask with 1× Trypsin-EDTA (Ethylenediaminetetraacetic acid) solution. The cell suspension was centrifuged at 1500 rpm for 5 min and then re-suspended in growth medium for further experiments.

NS Uptake Analysis by ICP-MS

The amount of silica NS present in the cells was determined by high-resolution sector field ICP-MS equipped with an autosampler. HeLa cells (3×10^5 cells/mL) were treated with 10 µL of each NS sample and incubated for 24 h. At the end of incubation, the cells were harvested by trypsinization, and then washed with 1×PBS several times to remove the debris. Cells were once more washed with HPLC grade water repeatedly and acid digested using 5 mL of mass spectrometric grade nitric acid (60%) for 30 min at 80°C, followed by dilution with water. The data were acquired at standard resolution using Indium (20 ppb) as an internal standard. The amount of relative incorporation of the NSs was calculated by using the formula: $x = [b/a] \times 100$

where x = amount (%) of relative incorporation inside the cell, a = amount given and b = amount incorporated.

MTT Assay

The effect of Si-NSs in proliferating the HeLa cells was studied using MTT assay. Briefly, cells (1×10^5 cells/well) were treated with increasing concentrations of the Si-NSs (0-45 ng/mL) followed by addition of 20 µL MTT solution (5 mg/mL) for 4h. The formed formazan crystals were dissolved in solubilization buffer and the optical density was measured at a wavelength of 570 nm using ELISA plate reader (Multiskan® EX, Thermo electron corporation). The cell viability ratio was calculated by the formula: Cell viability (%) = ($O.D._{\text{Si-NS treated cells}}/O.D._{\text{untreated cells}}$) × 100. Based on this the half-maximal inhibitory concentration (IC_{50}) value was ~5 ng/mL, which was used for further experiments.

Flow Cytometric Analysis

Cell Cycle Analysis: HeLa cells (3×10^5 cells/mL) were treated with 10 µL of each NS sample and incubated for 24 h. At the end of incubation, the cells were harvested by trypsinization and then washed with PBS. The cells were fixed in 70% ethanol and left overnight at -20°C. Finally, the cells were washed with PBS and incubated in staining solution (0.2 µg/mL PI, 0.2 µg/mL RNase A, 0.1% Triton X-100) for 2 h at 4°C in dark and analyzed by flow cytometry.

Measurement of ROS Generation: After treatment with different NS samples, in order to detect the generation of intracellular ROS in HeLa cells, 3×10^5 cells were collected, washed with PBS and re-suspended in (10 µM) dihydrodichlorofluorescein diacetate (H_2DCFDA). After 30 min of

incubation at 37°C, the fluorescence intensity was measured using flow cytometer at excitation and emission wavelengths of 488 and 520 nm, respectively.

Apoptosis Measurement: HeLa cells (3×10^5 cells/mL) were treated with 10 µL of each NS sample and incubated for 24 h. At the end of incubation, the cells were harvested by trypsinization, and then washed with PBS. Cells were incubated in staining solution (0.2 µg/mL PI, 0.2 µg/mL Annexin) for 2 h at 4°C in dark and analyzed by flow cytometry.

Microscopic Analysis

Overall surface morphology of the treated cells with respect to the control group was analyzed by FESEM. HeLa cells (3×10^5 cells/mL) were seeded onto round glass cover slips and maintained in 35 mm cell culture plates. Cells were treated with a different formulation of NS construction. After 24 h of treatment, cover slips were removed from the culture plate and washed with 1×PBS several times to remove the debris generated from cell rupture. Then the cells were fixed by 2% glutaraldehyde solution for 4 h at 4°C, washed again with 1×PBS and air dried. During FESEM analysis cover slips were coated with Au-Pd to improve the conductivity.

Fluorescence Microscopy

Hoechst-PI Staining for Live-dead Visualization: HeLa cells (3×10^5 cells/mL) were treated with different formulations of NS construction in 35 mm cell culture plate onto glass cover slips. After 24 h of treatment, cover slips were removed from the culture plate and washed with PBS several times and fixed by 2% glutaraldehyde solution. After cell fixation, post processing was done and cover slips were air dried. Dried cover slips were stained with (1 µg/mL) Hoechst-PI mix solution for 30 min. After 30 min cover slips were washed with 1×PBS for the removal of excess dye solution, then air dried and observed under a fluorescence microscope for the

detection of live/dead cells. Merged images of both channels were shown using a fluorescence microscope at 60× magnification.

TUNEL assay

TUNEL assay was performed to evaluate the chromosomal fragmentation analysis. The DNA fragmentation was detected by using *in situ* Cell Death detection Kit, AP (Roche Diagnostics; Germany) following the manufacturer's protocol, with slight modification. Briefly, after 24 h treatment by the NSs, cells were fixed by adding 2% glutaraldehyde at room temperature. The fixed cells were washed in 1×PBS repeatedly, permeabilized with 0.1% Triton X-100 for 5 min on ice, and then incubated with 50 µL of TUNEL reaction using terminal deoxynucleotidyl transferase and fluorescein-labeled dUTP for 60 min at 37°C in a humidified chamber in the dark. After labeling, the cells were washed in 1×PBS and counterstained with 2 µg/mL PI stain to visualize total DNA. The fluorescence obtained from the cells with damaged or fragmented DNA strand breaks were investigated under a fluorescence microscope.

DNA Isolation from Treated Cells for Classical DNA Ladder Assay

HeLa cells (3×10^5 cells/mL) were maintained in 35 mm cell culture plates. Cells were treated with a different formulation of NSs for 24 h. After 24 h of treatment the cells were harvested by trypsinization and washed with 1×PBS. Harvested cells were used to isolate genomic DNA by a standard protocol. Briefly, culture media was removed after 24 h of treatment. Cells were washed with 1×PBS and lysis buffer [67 mM Tris-HCl (pH 8.8), 16.6 mM ammonium sulphate, 5 mM β-mercaptoethanol, 6.7 mM magnesium chloride, 6.7 µM EDTA (pH 8.0), 1.7 µM sodium dodecyl sulfate (SDS) and 50 µg/mL proteinase K] was added into the cells and incubated for 1 h at 37°C, followed by a 10 min incubation at 80°C to inactivate the proteinase K. Cells were then

centrifuged at 8000 rcf for 2 min. Isolated genomic DNA was resuspended in Tris-EDTA (TE) buffer and 10 mg/mL of DNase-free RNase for 30 min at 37 °C. Finally, the extracted genomic DNA was loaded and fractioned on 2% agarose gel to get the pattern of DNA laddering.

Antibody Conjugation for Targeted Killing of Cancer Cells

HeLa and HEK-293 cells (1×10^4) in 35 mm culture plate were seeded overnight and then treated with the antibody conjugated NSs for 24 h. After treatment, the cells were washed with PBS for the removal of excess media and cell debris. Washed cells are then fixed by using 2% glutaraldehyde solution for 10 min. After removal and washing of glutaraldehyde by PBS, the cells were permeabilized by PBST (PBS containing 0.2% Tween 100) for 15 min at 4°C. The cells were then treated with Alexa Fluor® 488 conjugated secondary antibodies (1:5000 dilutions) for 4 h. After secondary antibody incubation, cells were washed with PBS for the removal of excess antibody. Cells were then counter stained with DAPI (300 nM) for 5 min and washed with PBS and air dried in dark before imaging. Cover slips were fixed and mounted onto the glass slides with DPX. The slides were visualized under an inverted fluorescence microscope and the photographs were taken by excitation with a blue and green filter.

Hierarchical Cluster Analysis

Hierarchical cluster analysis was performed employing SPSS 21 for windows, on data obtained as mean ± standard deviation of the number of independent experiments.

Molecular Dynamics Simulation

The all atomistic molecular dynamics (MD) simulation was carried out using Nanoscale Molecular Dynamics (NAMD) program, and visualized in Visual Molecular Dynamics (VMD) software package. For the construction of the molecular structure of silica NSs, at first silica NS

was made from inorganic builder tool of VMD and then replicated to the preferred size dimension pertaining to the AFM measurements where the NSs of different thicknesses share common X and Y dimensions but differ only in the dimension along the Z-axis. The finally constructed NSs have 2400, 4800, 7200 and 9600 atoms for U1, U3, U5 and U7, whereas the average thickness was kept at 0.4, 1.4, 2.6 and 3.3 nm, respectively. The cell membrane containing 117440 atoms in a volume of $10 \times 10 \times 5$ nm^3 was made from a membrane builder tool present in VMD. The size dimension of the cell membrane was kept constant in all simulations. All NSs were allowed to undergo two equilibration stages in order to integrate their amorphous property. In the first step, the system temperature was increased to 8000 K from 310 K, and in the second step of equilibration the temperature of the system again cooled down to room temperature. Our simulated silica NSs have almost 10 times smaller thickness (*i.e* dimension of Z-axis) than the observed AFM parameters. In our simulation study, we kept the initial X-Y dimension constant at almost 110×50 Å^2, as compared to the experimentally observed lateral dimensions of $\sim 100 \times 200$ nm^2 from AFM measurements. The NSs were then positioned at different orientations with respect to the membrane. In every orientation the distance between Si-NS and the membrane was kept constant at ~6 Å, to facilitate the primary non-bonded interactions. Here, we first oriented NS's Z-plane perpendicular to the membrane's X-Y plane. Similar orientation was followed for all the corresponding NSs of different thicknesses. Next we tested our hypothesis of the sharp edge cutting of NS by orienting only the thinnest NS in two different ways. In the first type, NS's X-Y plane was placed on the membrane's X-Y plane horizontally. In another configuration a corner tip of the NS was placed on the membrane's X-Y plane. All NS constructs were kept at 90° against the membrane leaf, except for the diagonal orientation of U1 NS (U1-D)U1-D where the contact angle was set at

45°. After configuring the spatial arrangements, the system was partially hydrated to stabilize the membrane structure only. Before the final production run of 3×10^5 steps, the system was allowed for 1×10^5 steps of energy minimization. The simulations were performed under number-pressure-temperature (NPT) ensemble (at a temperature of 298 K and pressure of 1 atm) to control the simulation temperature and pressure, whereas CHARMM22 force field was used. The interaction energy was calculated by the in built NAMD energy calculation tool.

Zeta Potential of Si-NSs

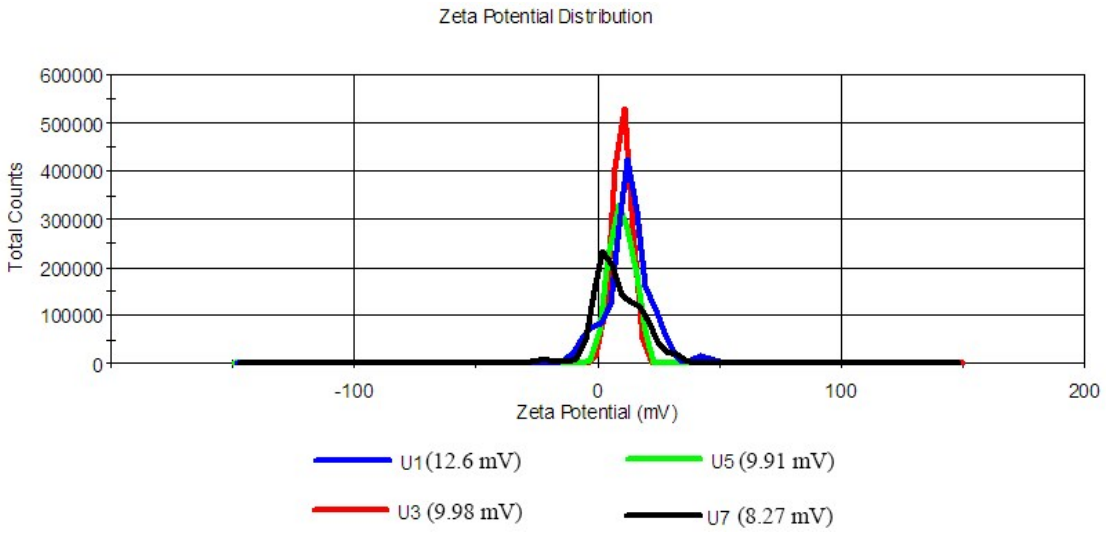


Fig. S1: Graphical representation of zeta potential values of unmodified NSs of different thickness.

Table S1: Loading concentration of ligand molecules on the Si-NSs.

Ligand	D1	F1	DF1
Doxorubicin (ng/µg of Si-NS)	~285	NA	~335
Folic acid (ng/µg of Si-NS)	NA	~375	~347

Height Profiles of D1, F1 and DF1 NSs

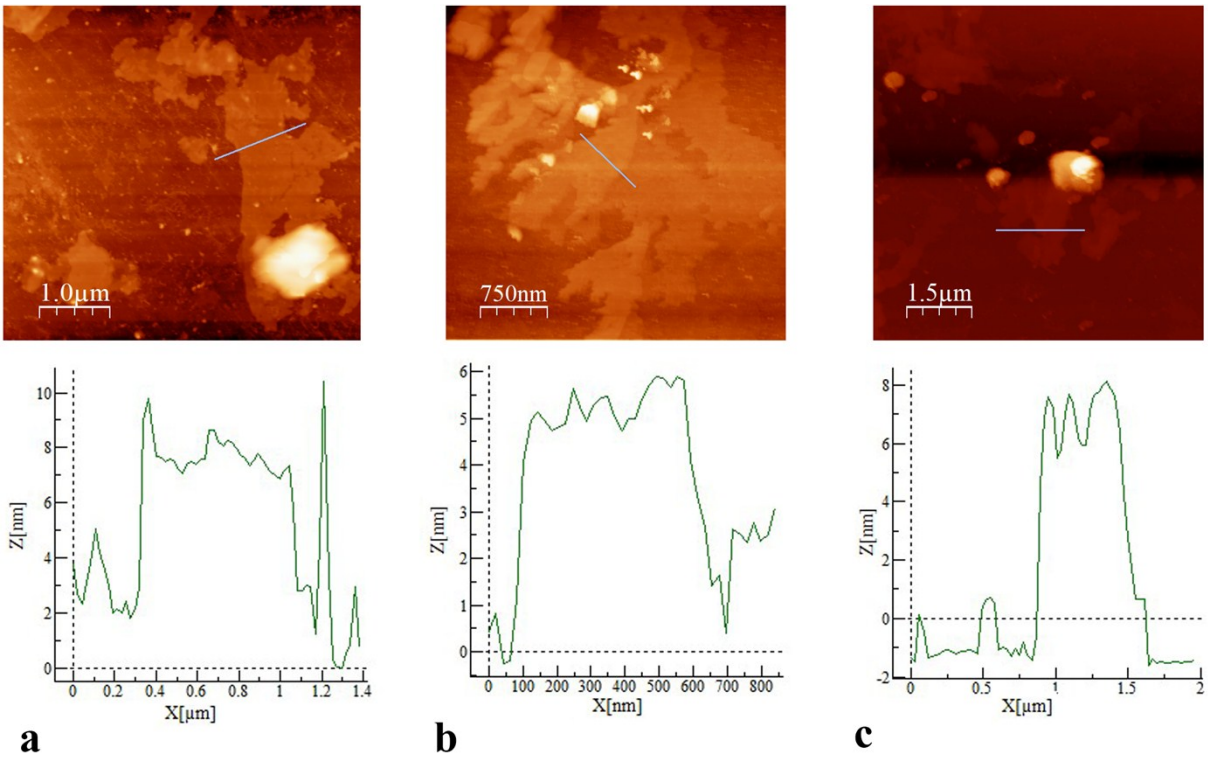


Fig. S2: AFM images (top panel) along with height profiles (bottom panel) of (a) D1, (b) F1 and (c) DF1 NSs. Due to different modifications, the thickness of the U1 NSs increases from 2.9 ± 0.01 nm to 8.5 ± 0.04 nm for D1, 5.3 ± 0.5 nm for F1 and 7.2 ± 0.3 nm for DF1 NSs.

TEM Image of U3 NSs

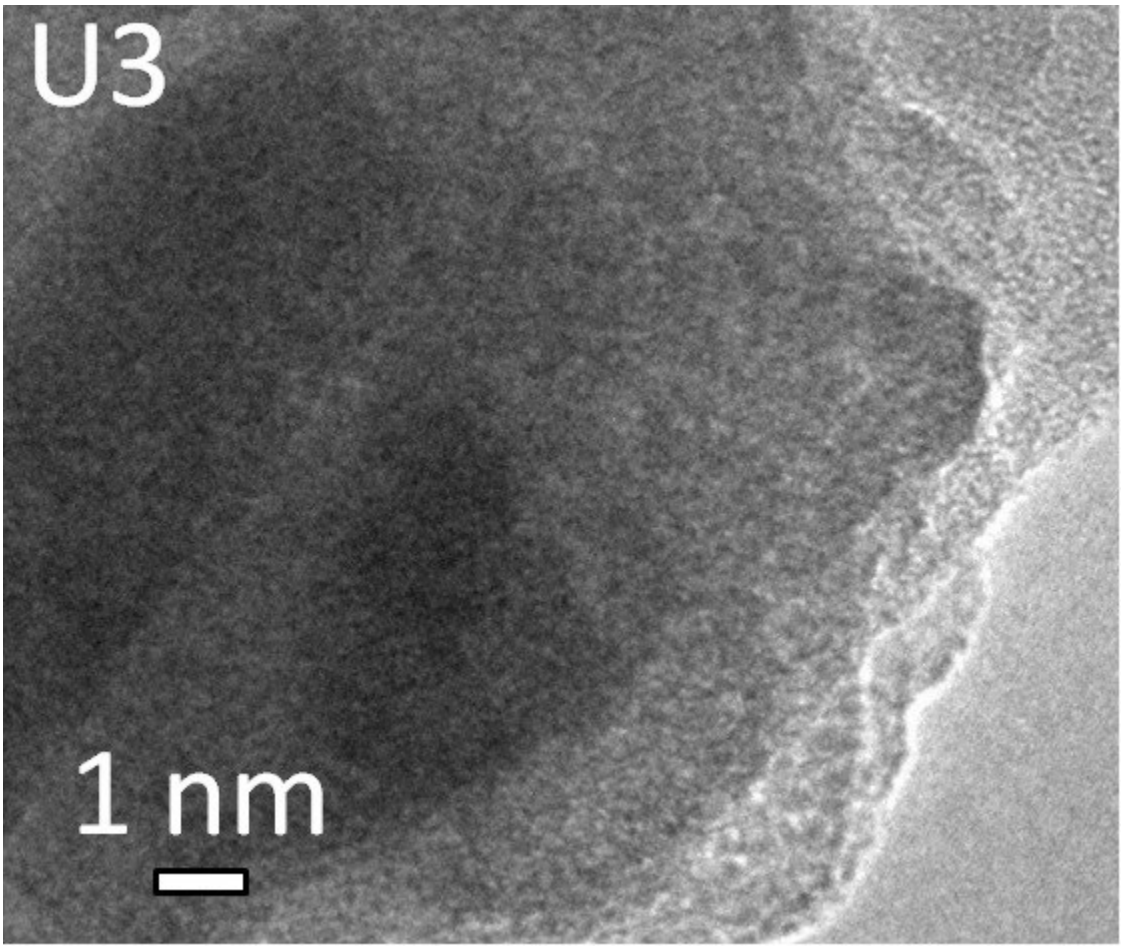


Fig. S3: TEM image showing the stacking of U3 NSs.

FTIR Studies

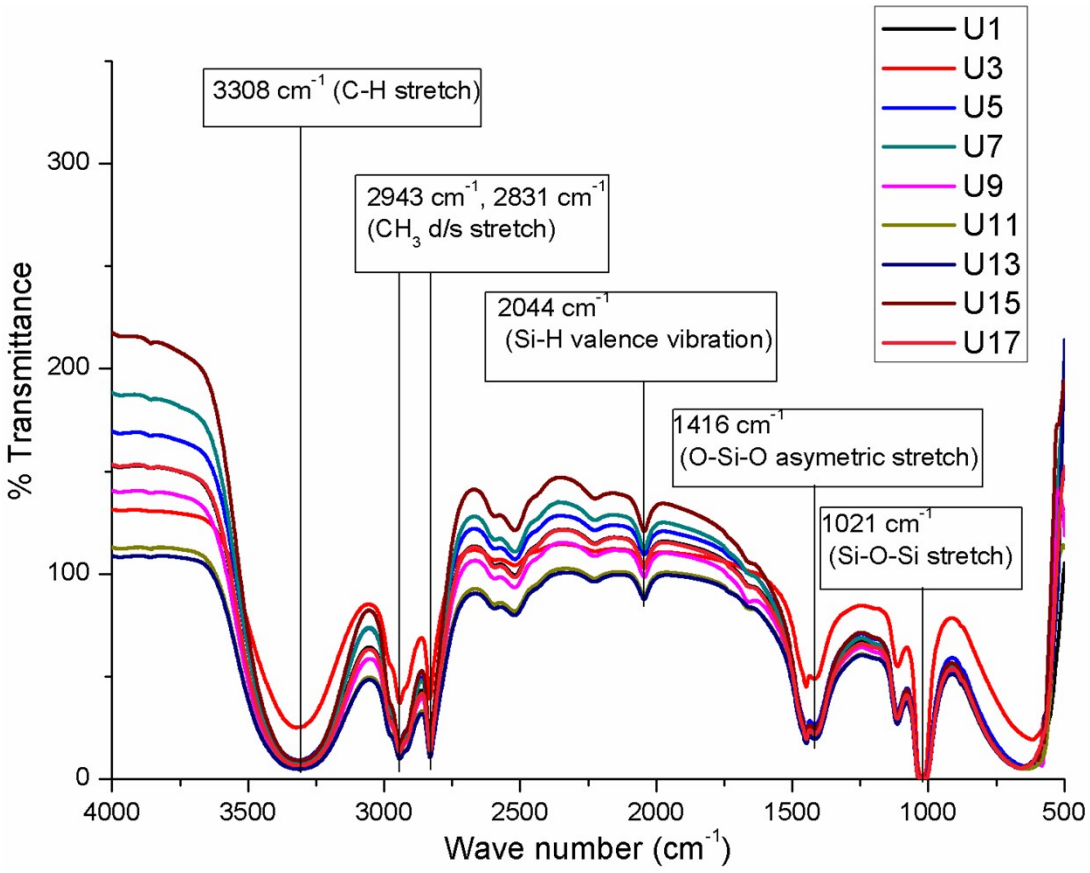


Fig. S4: FTIR spectra of unmodified NSs of different thicknesses. No significant change is observed in the spectra indicating unchanged bonding pattern due to NS self-stacking. U(Number) denotes the NSs self-stacked over the number of days.

A majority of the vibrational bands are due to the presence of CTAB and methanol, which has been used as surfactant and solvent, respectively. The bands at 3308, 2943 and 2831 cm^{-1} correspond to C-H, CH_3 d/s stretching frequencies, respectively. A series of other peaks can be attributed to the presence of the Si-related species, *e.g.* 2044 cm^{-1} (Si-H valence vibration), 1416 cm^{-1} (O-Si-O asymmetric stretch) and 1021 cm^{-1} (Si-O-Si stretch). In general siloxanes show strong absorption in the range of 1130-1000 cm^{-1} and with elongation of the chain or its branching, the peak broadens and becomes more complex by the presence of two or more overlapping bands. In our case, the NSs show a shift of transverse optical (TO) and longitudinal optical (LO) peaks towards higher wave numbers. The original position of these two peaks is 1065 cm^{-1} (TO) and 1252 cm^{-1} (LO).

Elemental Mapping of U3 NSs

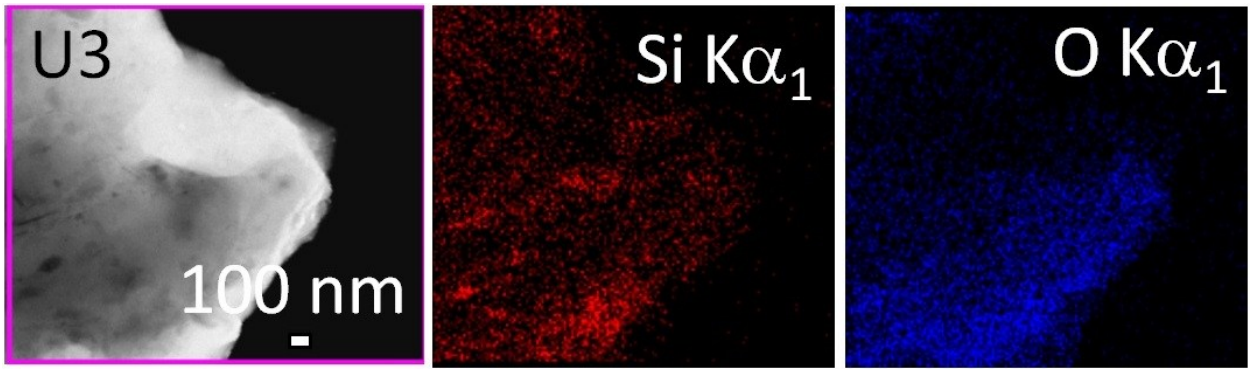


Fig. S5: HAADF-STEM image (left panel) and EDS mapping of U3 NSs.

ICP-MS Analysis of the Incorporation of Unmodified and Modified NSs inside HeLa Cells

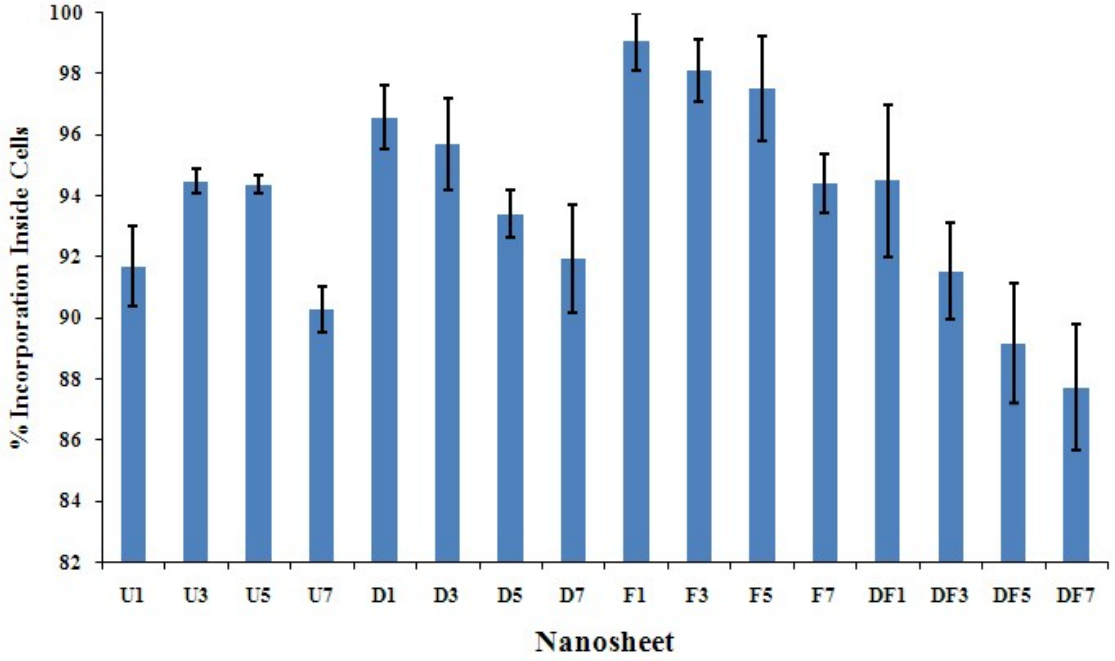


Fig. S6: The percent incorporation of the unmodified and modified NSs inside the HeLa cells, measured by ICP-MS. The results markedly demonstrate the higher intracellular abundance of folic acid modified NSs.

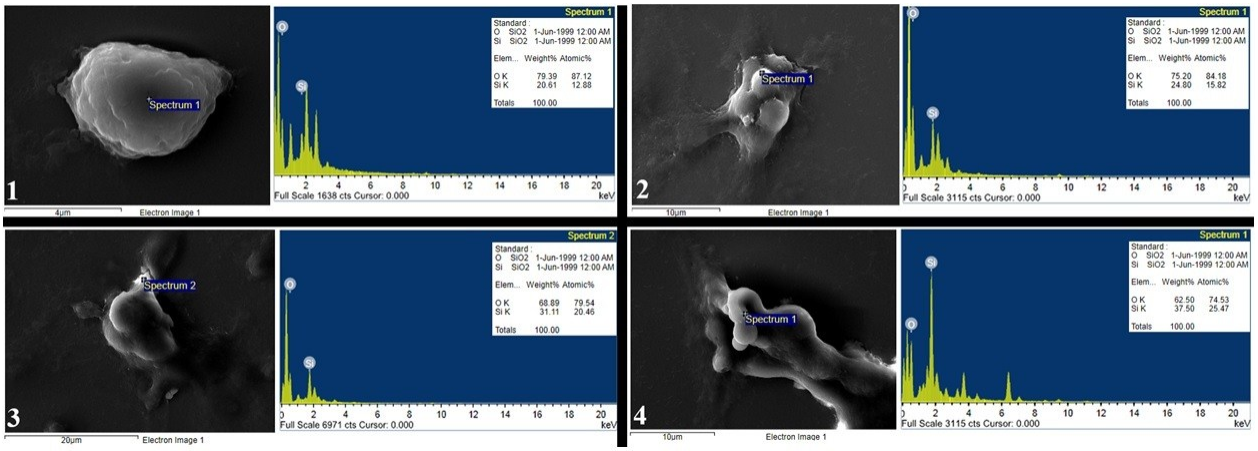


Fig. S7: The temporal incorporation of U1 NSs inside the HeLa cells investigated by monitoring the Si wt% from EDS analyses. The FESEM image and EDS spectrum for (1) untreated cells, and treated cells after (2) 2, (3) 4 and (4) 8 h.

Morphology of Cell Membrane Exposed to the Thicker NSs

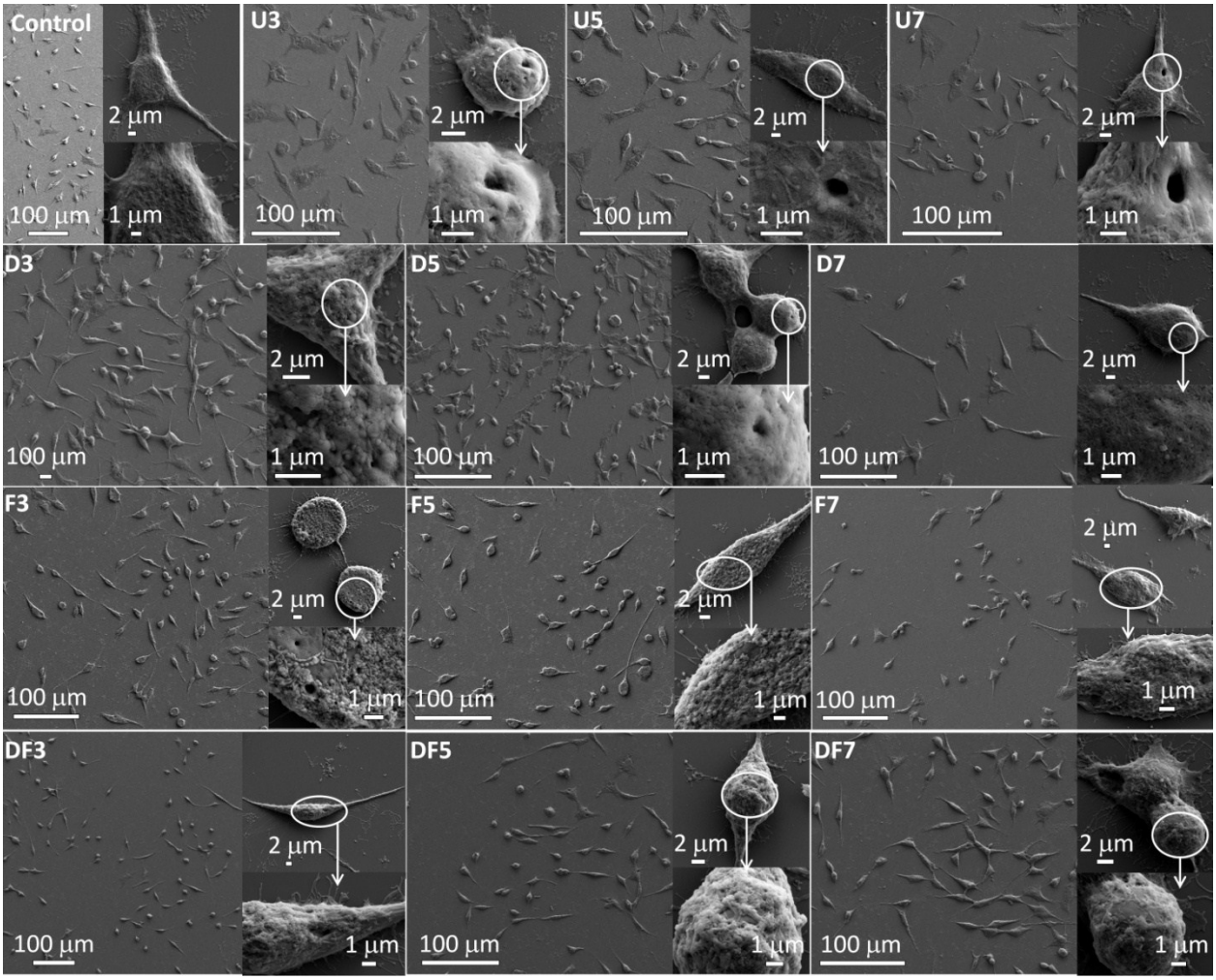


Fig. S8: FESEM images of the cells after treatment with the thicker NSs with different formulations. Upper insets show the magnified cells and lower insets represent their surface. The pores created by the NS ingress can be visualized by the darker contrasts in the lower insets.

Epifluorescence Microscopy

HeLa cells were cultured for 24 h under standard conditions and then treated for 8 h with U1 NSs. Before fixation, the treated cells were treated with CellLight plasma membrane stain in manufacturer recommended protocol and visualized under epifluorescence microscope.

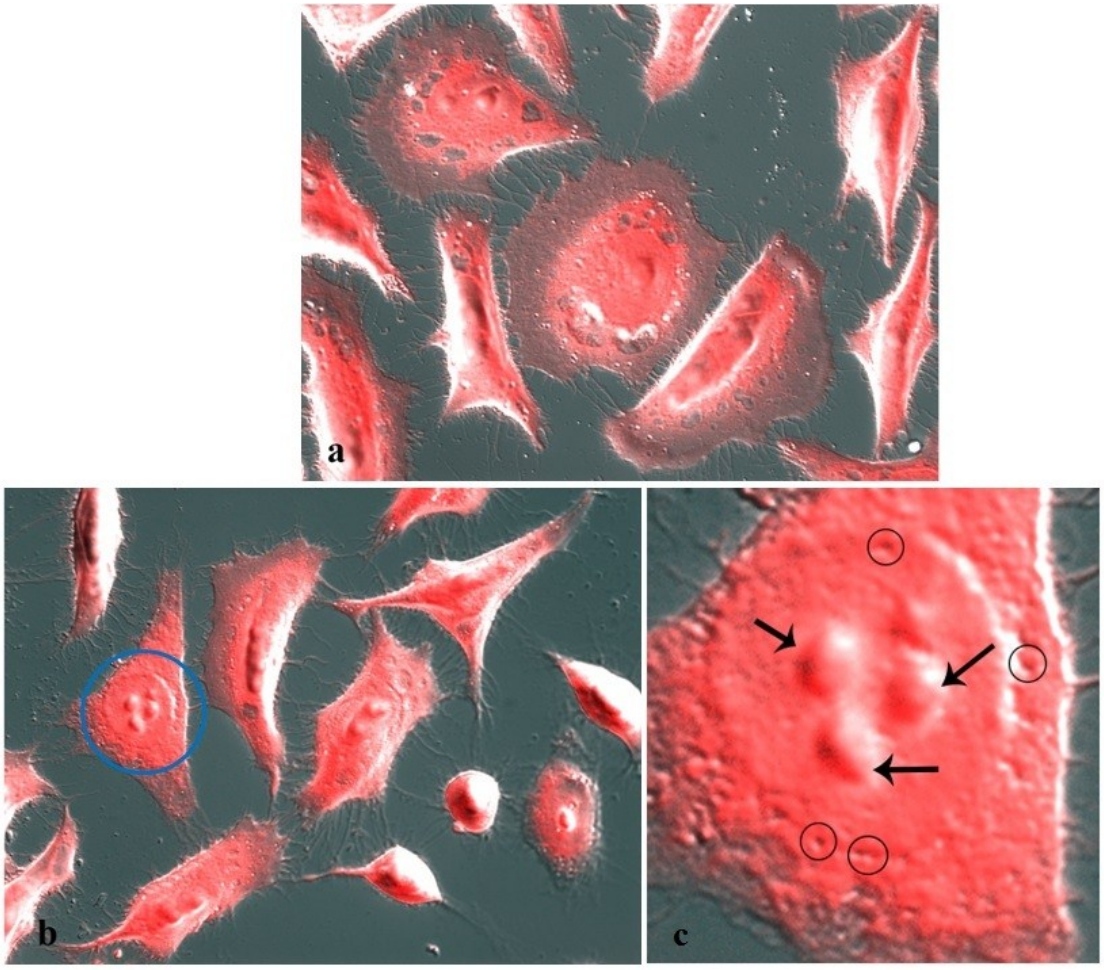


Fig. S9: Epifluorescence microscopy of (a) control HeLa cells before treatment with the NSs, and (b) U1 NS treated cells post 8 h treatment. (c) Enlarged view of the circled part of (b) showing nuclear degranulation (black arrows) and formation of pores (circled) due to U1 NS treatment.

Molecular Dynamics Simulation Images of the Spontaneous Ingress of Unmodified Thicker Si-NSs

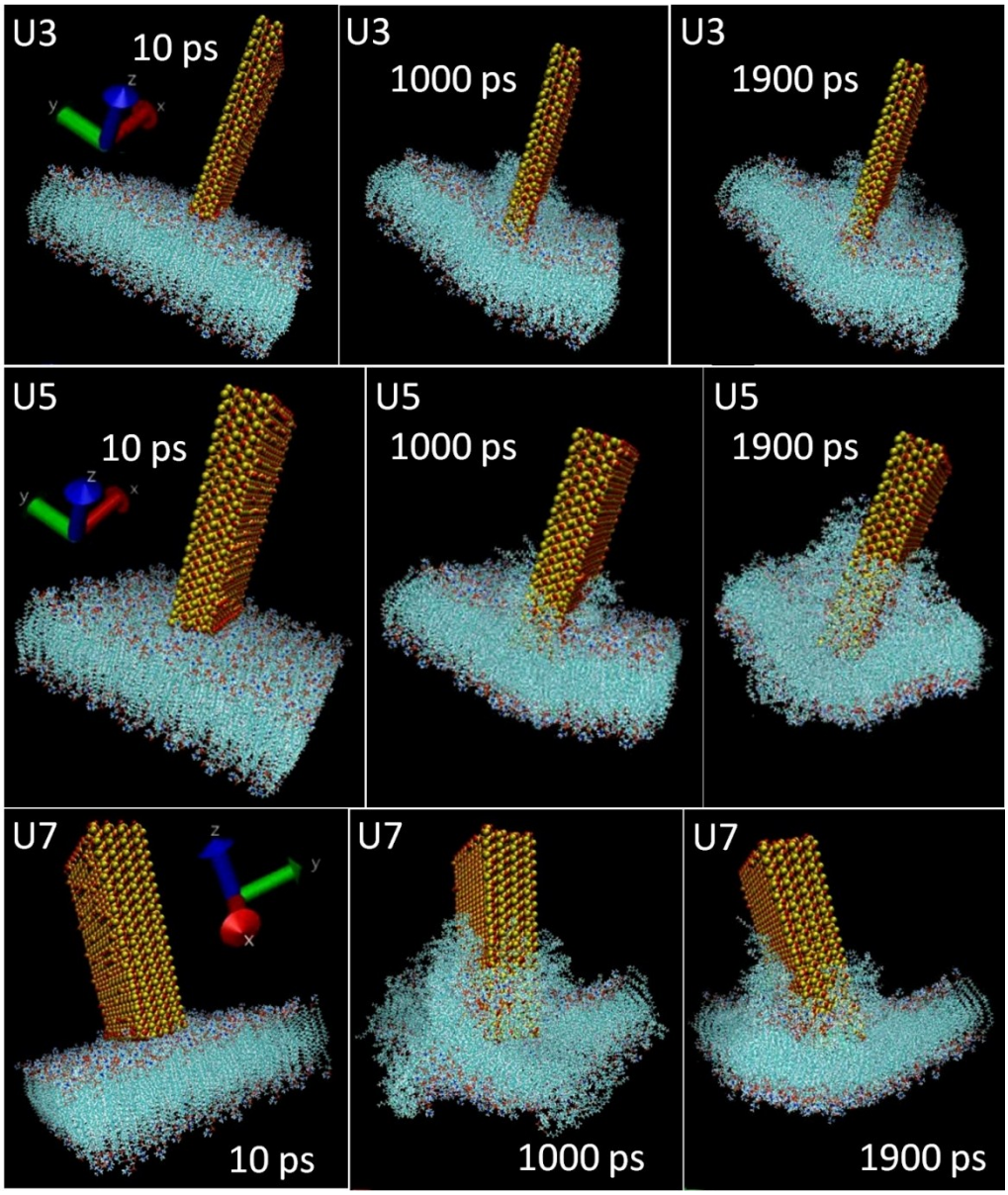


Fig. S10: Simulated insertion profiles of U3, U5 and U7 NSs in a vertical configuration docked at the outer leaf of a phospholipid bilayer (POPC) and its extraction. The snapshot times are indicated in the respective panels. The atomic colour codes are: cyan (carbon), white (hydrogen), red (oxygen), dark blue (nitrogen), orange (phosphorous), and yellow (silicon). The NSs are shown to be crystalline as yellow-red sheets.

Tilt Angle of the NSs obtained from MD Simulations

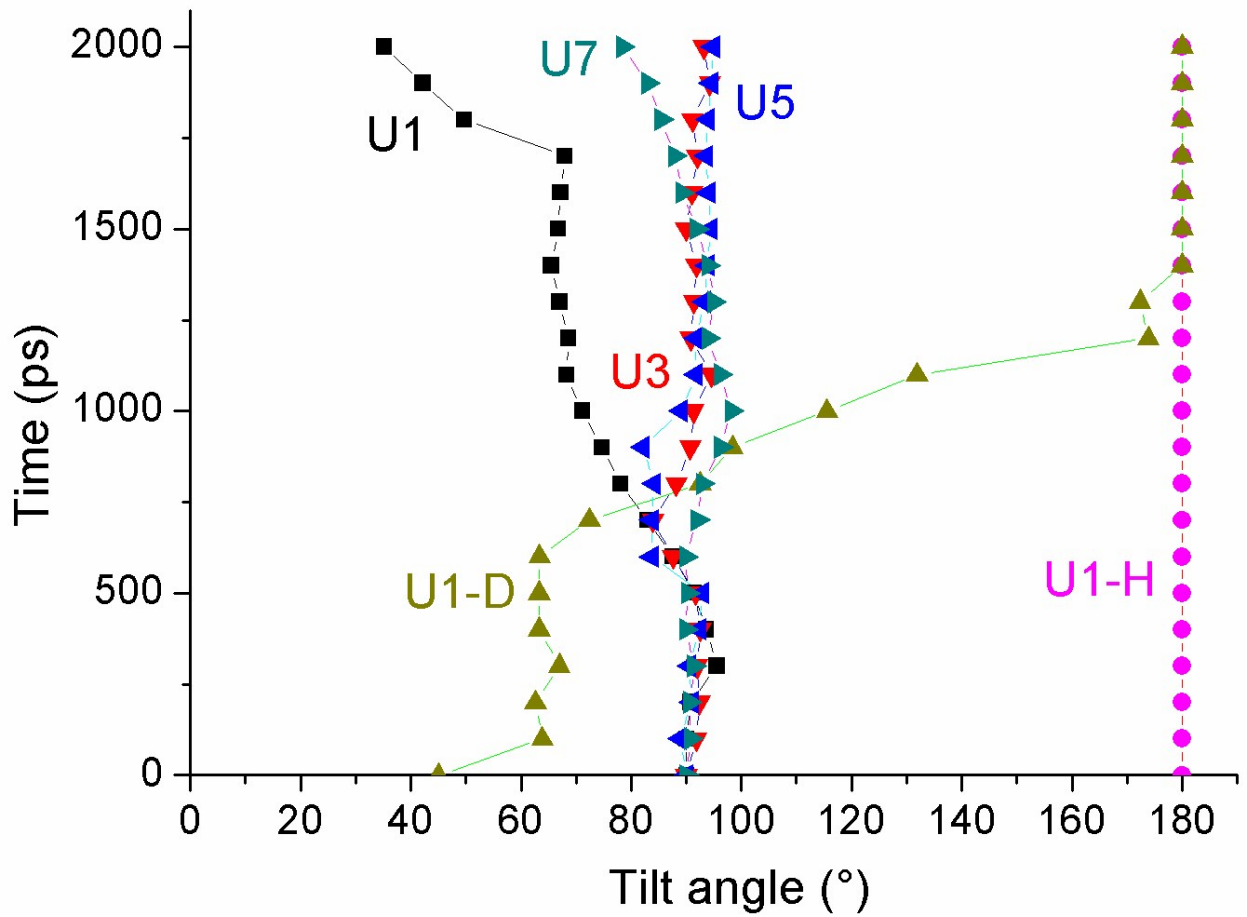
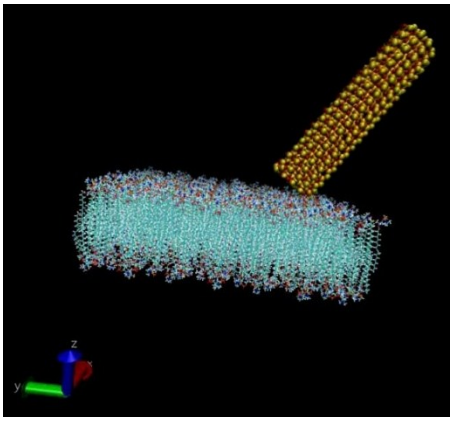
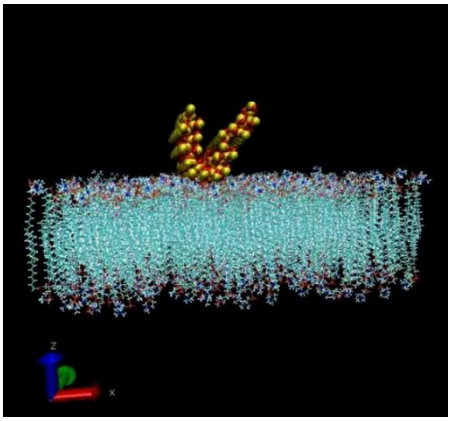


Fig. S11: The tilt angle of different NSs measured as a function of time from the MD simulations. The number of data points are kept minimal to preserve clarity of the plots.

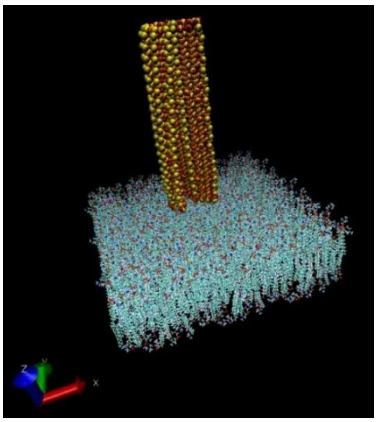
Discussion S2: Movie File Legends with Still Images from MD Simulations



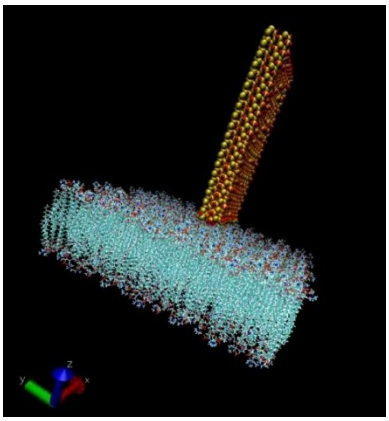
Movie S1-(Day1-diag): Phospholipid extraction by U1 NS oriented diagonally to the outer leaf of phospholipid bilayer.



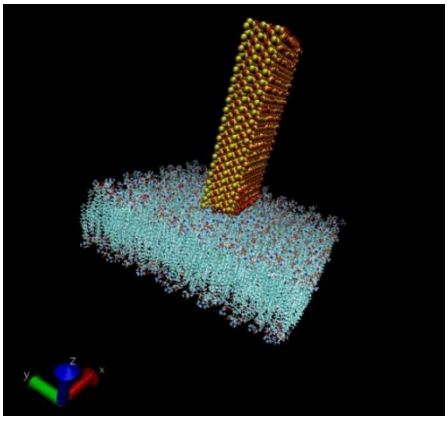
Movie S2-(Day1-horiz): Phospholipid extraction by U1 NS oriented horizontally to the outer leaf of phospholipid bilayer.



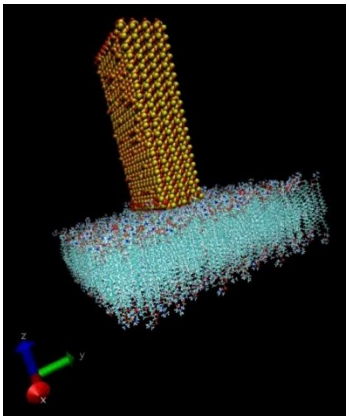
Movie S3-(Day1): Phospholipid extraction by U1 NS oriented vertically to the outer leaf of phospholipid bilayer.



Movie S4-(Day3): Phospholipid extraction by U3 NS oriented vertically to the outer leaf of phospholipid bilayer.



Movie S5-(Day5): Phospholipid extraction by U5 NS oriented vertically to the outer leaf of phospholipid bilayer.



Movie S6-(Day7): Phospholipid extraction by U7 NS oriented vertically to the outer leaf of phospholipid bilayer.

Apoptosis Assay with Hoechst and Propidium iodide (PI)

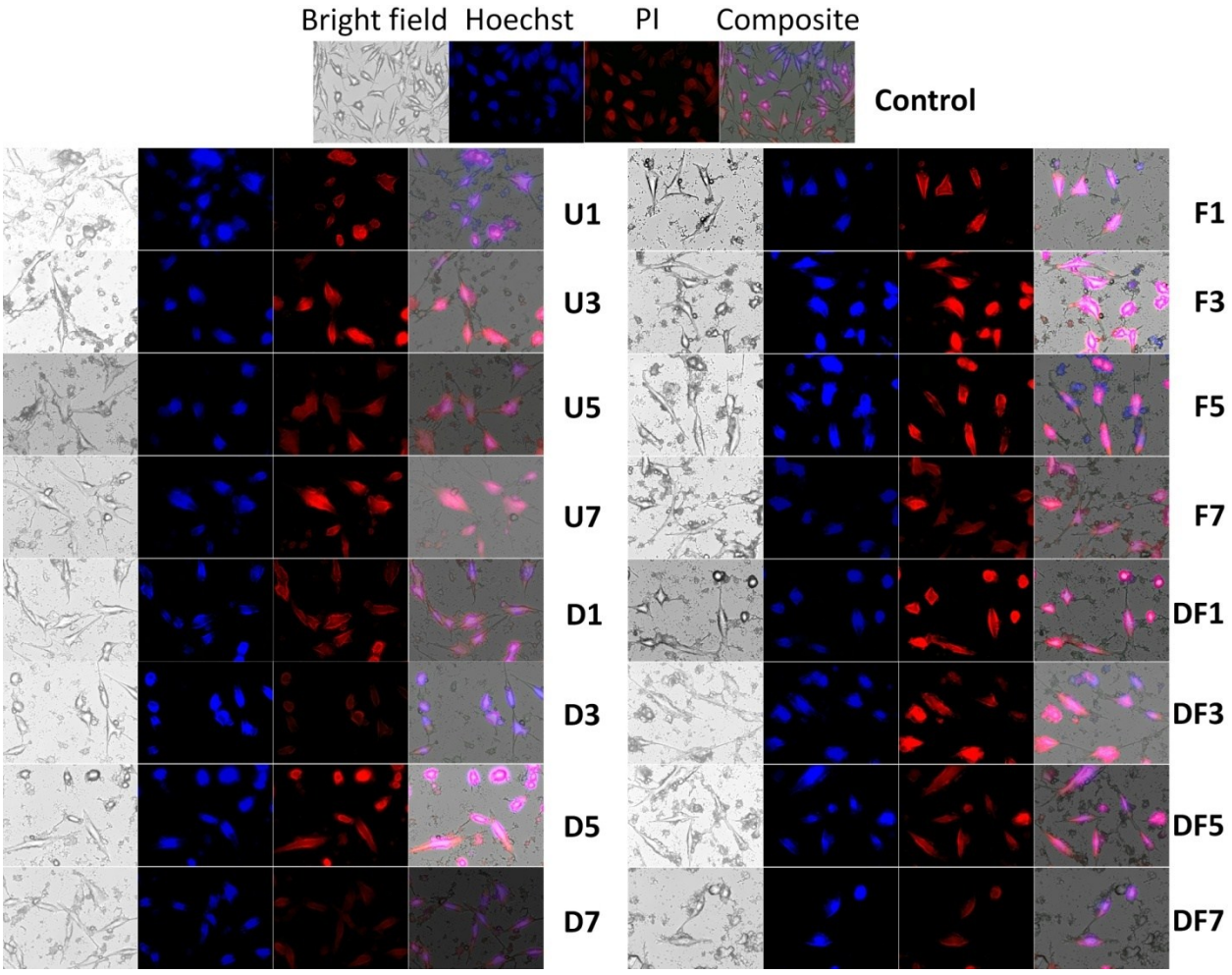


Fig. S12: Hoechst-PI co-staining of the treated HeLa cells showing the effect of the NS formulations. A significant change in fluorescent intensity occurs when the surface of the material is modified with folic acid.

TUNEL Assay of HeLa Cells Treated with Unmodified and Formulated Thicker NSs

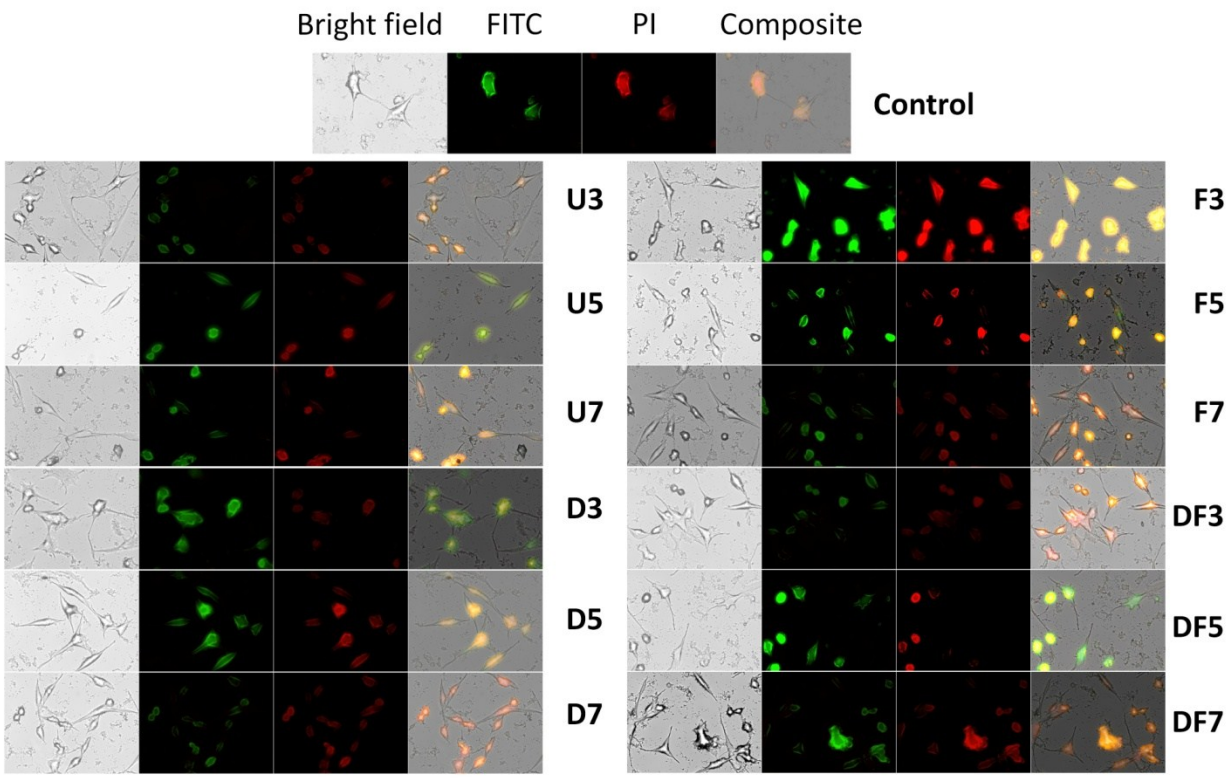
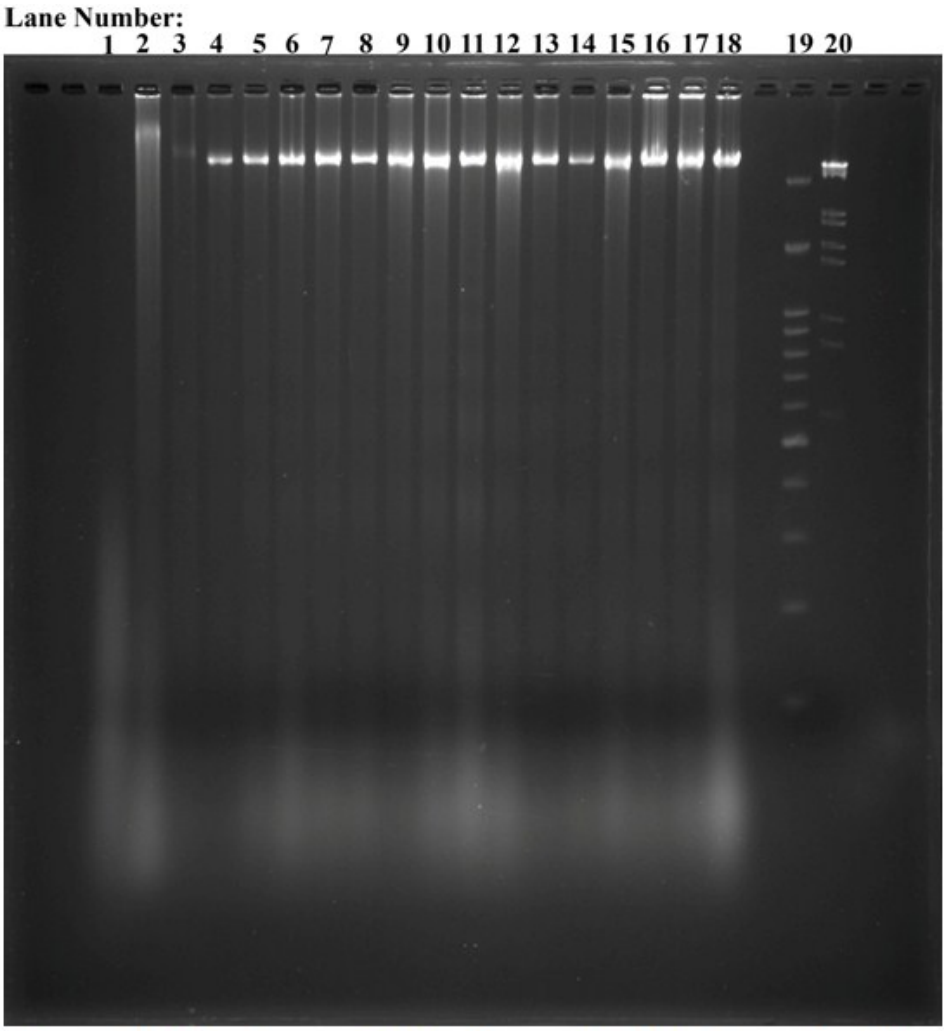


Fig. S13: TUNEL assay of the treated HeLa cells showing the effect of the thicker NS formulations. Higher the fluorescent intensity higher is the extent of DNA breakage.

DNA Ladder Test



Lane 1: U1; **Lane 2:** U3; **Lane 3:** U5; **Lane 4:** U7; **Lane 5:** D1; **Lane 6:** D3; **Lane 7:** D5; **Lane 8:** D7; **Lane 9:** F1; **Lane 10:** F3; **Lane 11:** F5; **Lane 12:** F7; **Lane 13:** DF1; **Lane 14:** DF3; **Lane 15:** DF5; **Lane 16:** DF7; **Lane 17:** Untreated control; **Lane 18:** Treated control; **Lane 19:** 100 bp DNA Ladder; **Lane 20:** Lambda DNA Ladder

Fig. S14: Classical in gel laddering test in agarose gel with the isolated genomic DNA from the treated samples.

Antibody Conjugated U1 NSs

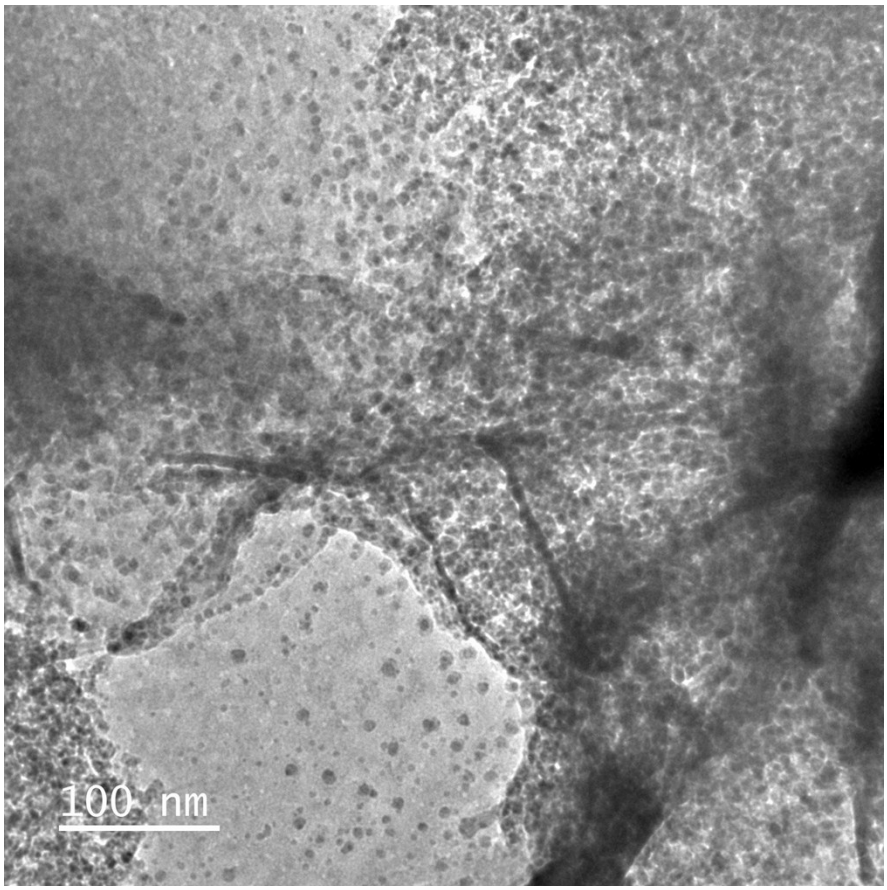
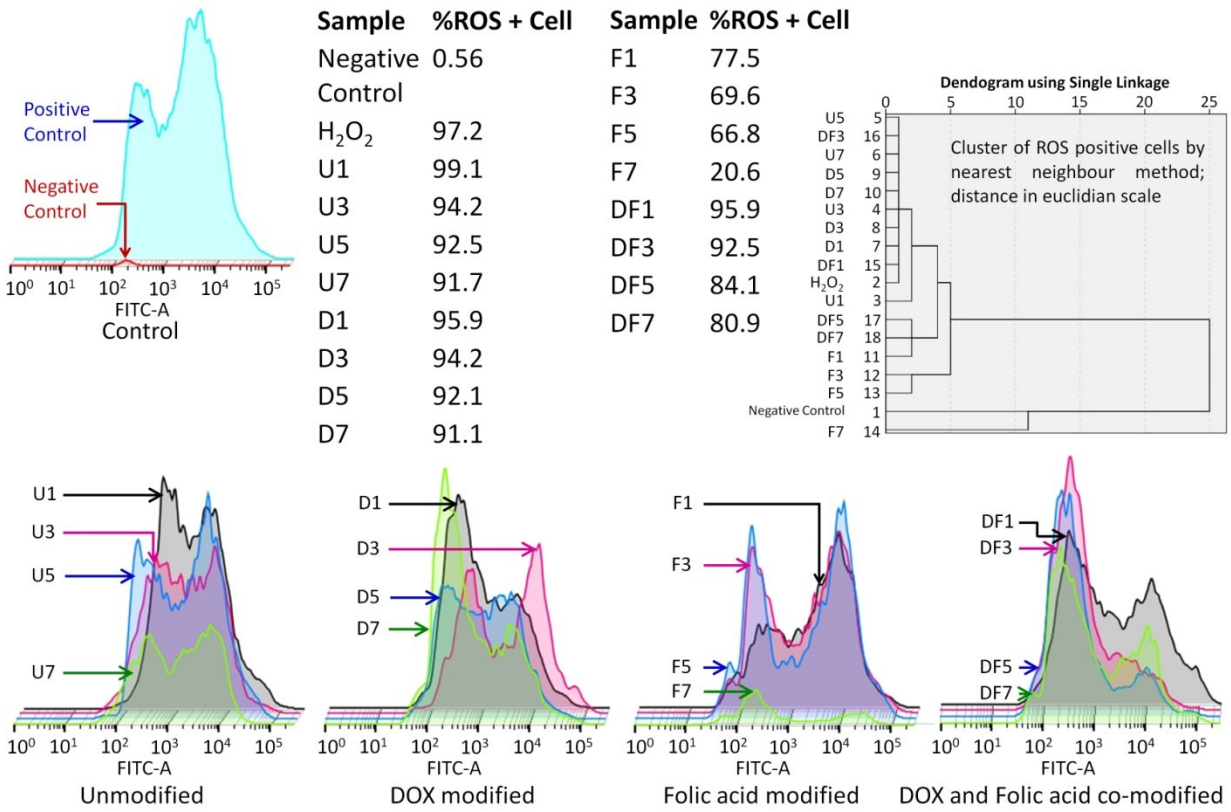


Fig. S15: TEM image of antibody conjugated NSs (U1+A).

ROS Generation Inside HeLa cells after treatment with Unmodified and Thicker Silica NSs



Sample	%ROS + Cell	Sample	%ROS + Cell
Negative Control	0.56	F1	77.5
H_2O_2	97.2	F3	69.6
U1	99.1	F5	66.8
U3	94.2	F7	20.6
U5	92.5	DF1	95.9
U7	91.7	DF3	92.5
D1	95.9	DF5	84.1
D3	94.2	DF7	80.9
D5	92.1		
D7	91.1		

Fig. S16: ROS generation. The bottom panel shows the typical flow cytometric data of ROS generation inside the HeLa cells after treatment with U/D/F/DF group of NSs. The control group (left panel) consists of positive control (blue) i.e. the cells treated with H_2O_2 and negative control (red) is without any treatment. The right panel shows the hierarchical clustering of the percentage of the ROS+ cells obtained from flow cytometric analysis. The table shows the ROS generation with different nanoformulations and the attenuation of sub-cellular ROS level by folic acid modified cells.

Cytotoxicity Analysis of Different Si-NSs

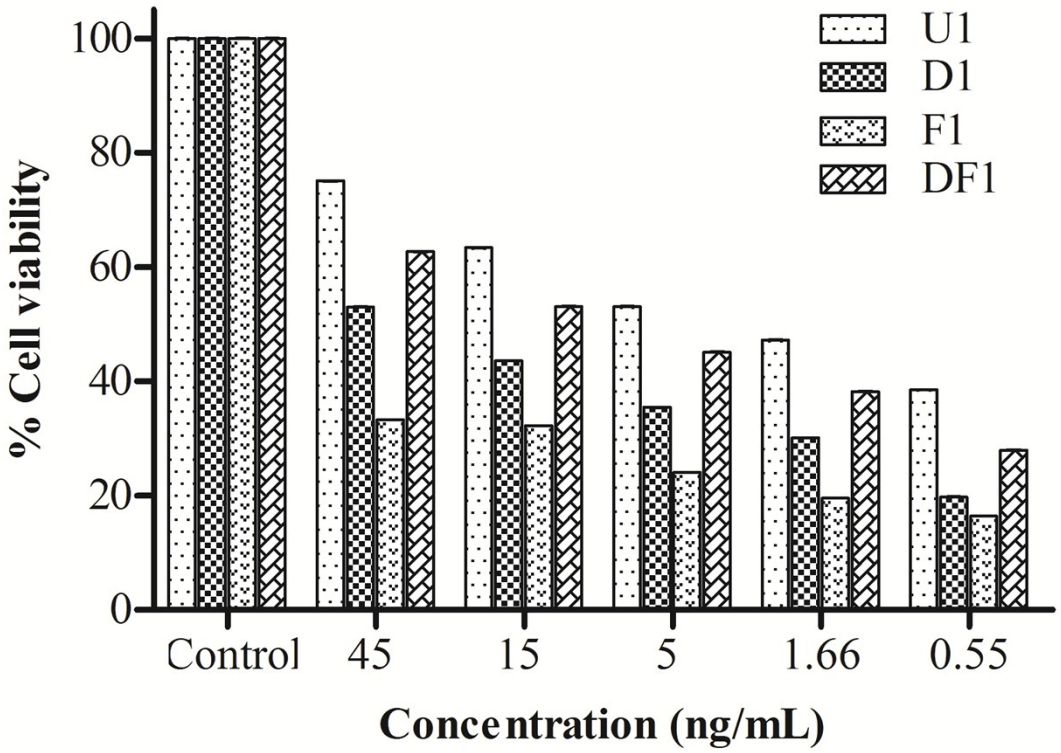
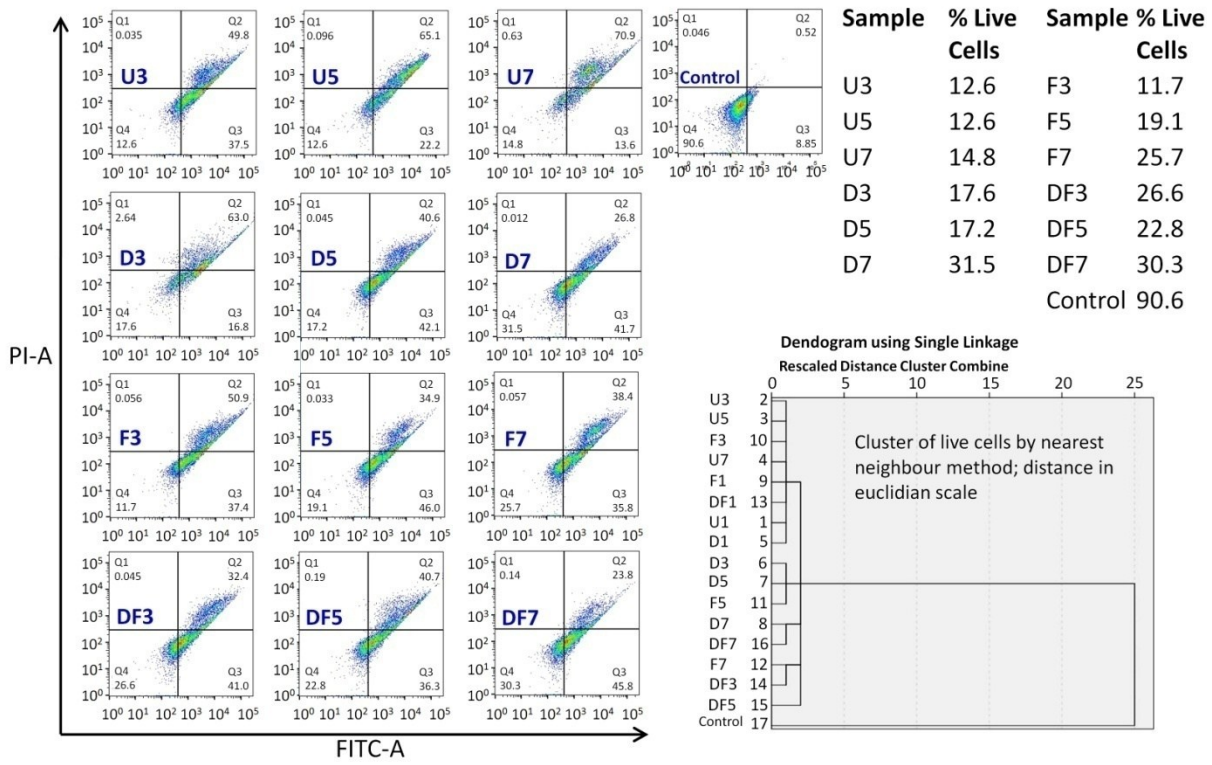


Fig. S17: Cytotoxicity analysis by MTT on different Si-NS treated HeLa cells.

Considering the percentage of incorporation of different NSs, F1 NSs have maximum incorporation into the cells (Fig. S6) and hence it can be speculated to show maximum toxicity by the production of ROS. But, the amount of generated ROS is comparably low in the case of F1, since folic acid actively scavenges the ROS (Fig. 6a). On the other hand, live cell percentage is quite low in the case of F1 (7.03%; Fig. 6b). In fact DF1 NSs have a similar consequence and in both cases, folic acid plays a crucial role to recognize the cell surface at a higher proficiency. Upon treatment with DF1, folic acid actively takes part in cell recognition and DOX kills the cells besides the scalpelling effect of the NSs. Although the MTT assay expectedly shows the least cell viability with F1 NSs, a primary toxicity test relies on the conventional biochemical pathway which might be misleading by deviating the conclusions far from the true mechanism of killing by sharp edge ingress.

Live cell Percentage after Insertion of Thicker NSs



Sample	% Live Cells	Sample	% Live Cells
U3	12.6	F3	11.7
U5	12.6	F5	19.1
U7	14.8	F7	25.7
D3	17.6	DF3	26.6
D5	17.2	DF5	22.8
D7	31.5	DF7	30.3
		Control	90.6

Fig. S18: (Left panel) Annexin-PI staining followed by flow cytometric analysis on treated cells. In all cases, Q1, Q2, Q3 and Q4 represent necrotic cell amount, late apoptotic, early apoptotic and live cell percentage, respectively. PI-area (*y*-axis) is plotted against Annexin-area (FITC-A) (*x*-axis). (Right) Hierarchical clustering of the percentage of the live cells obtained from flow cytometric analysis using Annexin-PI. DF NSs have a negative effect on cancer cell death while F3-F7 NSs demonstrate a positive effect on cell death.

Cell Cycle Analysis

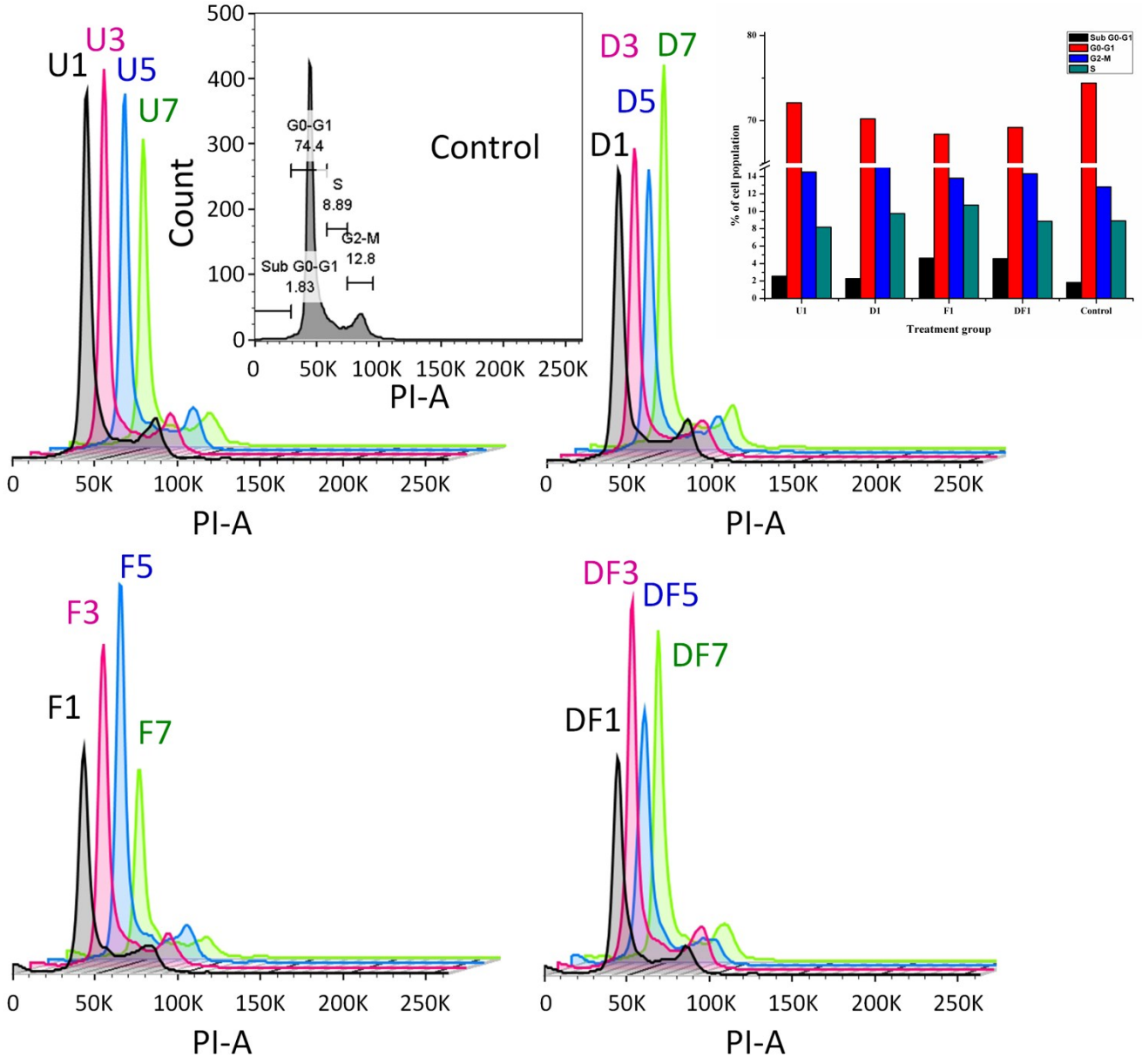


Fig. S19: Cell cycle analysis by flow cytometry of treated cells showing that the nano-formulations do not have any significant effect on the cell cycle regulation.