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pH Sensitive Coiled Coils: A Strategy for Enhanced Liposomal Drug Delivery

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Stimuli responsive controlled release from liposome based vesicles is a promising strategy for the site specific delivery of drugs. Herein, we report the design of pH sensitive coiled coils and their incorporation into the liposome as triggers for the controlled release of encapsulated drugs. The designed coiled coil peptides with the incorporation of environment sensitive fluorescent amino acids were found to be stable at physiological pH and unstructured while changing the pH of the environment to either acidic or basic. This pH dependent conformational switch of the coiled-coil polypeptides was exploited as triggers for the enhanced release of the encapsulated drug molecules from liposomes. The SEM, DLS and TEM analysis revealed the uniform morphology of the peptide liposome hybrid vesicles. Further, the drug encapsulated liposome internalization experiments with cancer cells revealed the enhanced release and accumulation of drug in the acidic lysosomal compartments in comparison with liposome without coiled coils.

Introduction

Nanotechnology based site specific delivery systems have attracted considerable attention in recent years due to their improved efficacy and reduced toxicity of potent therapeutics in the treatment of various diseases. 1 Liposomes have been serving as one of the major class of cargo delivery systems. 2 In this regard, several strategies were developed to increase the accumulation of drugs into the tumor site by the help of the enhanced permeability and retention effect (EPR) and binding to the tumor specific receptor. 3 Although these strategies are very attractive from the perspective of cancer treatment, the main drawback of the liposomal delivery is the lack of ability to actively release the entrapped bioactive molecules. 4 These aforesaid delivery systems depend primarily on either passive diffusion or slow non-specific degradation of liposomal carrier. 4 To overcome these shortcomings of liposomes, several strategies have been developed in recent years to release the entrapped drugs and bioactive molecules using external triggers including light, temperature, pH, and target specific enzymes. 5 In addition, Sung et al reported the carbon dioxide bubble generating liposome delivery through the use ammonium carbonate. 6 Among the various strategies, temperature and pH sensitive peptide triggers have gained considerable attention. 7 Recently

Scheme 1: Helical wheel diagram of coiled coil peptides. Letters X and Z are representing the position of the fluorescent amino acids in the designed coiled coils.

Scheme 2: Sequences of the coiled coil peptides.
Kostarelos and colleagues\(^4\) and we reported the coiled coils as mild temperature triggers for the controlled release of encapsulated molecules from liposome.\(^5\) Coiled coils are very important super secondary structural motifs of protein structures.\(^6\) The coiled coils are primarily consists of seven-residue (‘heptad’) sequence repeats, denoted as “α-b-c-d-e-f-g”. Positions \(a\) and \(d\) are predominantly occupied by the hydrophobic amino acids and the polar residues generally occupied other positions. Specific intermolecular hydrophobic and ionic interactions of coiled coil peptide partners play pivotal role in the stability of coiled coil structures.\(^7\) Further, the conforamtional change of coiled coils with respect to the temperature, salts, and pH of the environment have been extensively investigated.\(^8\) Many heterodimeric coiled coils showed increased stability in low pH rather at physiological pH.\(^9\) Recently, Klok and colleagues studied pH triggered E3/K3 heterodimeric coiled coil which is stable at pH 7 and unfolded at pH 5.\(^10\) We anticipated that the coiled coil peptides which are stable at physiological pH and unfold at acidic pH can be used as triggers for intracellular drug release through the endosomal pathway. We envisioned that altering the coiled coil peptides through the incorporation of environment sensitive and bulky fluorescent amino acids\(^11\) at hydrophobic positions it can be possible to design pH sensitive coiled coils and they can be envetually used as triggers for the controlled release of encapsulated bioactive molecules from liposomes. The design was basically intended to take the advantage of acidic pH in the lysosomal compartment to trigger the conformational change of the coiled coils incorporated within bilayers of liposome. Herein, we are reporting the design of pH sensitive heterodimer coiled coils with environmemt sensitive fluorescent amino acids at the hydrophobic sites, their incorporation into the bilayer of liposome, their utility as pH sensitive triggers for the control release of entrapped drugs from the peptide liposome hybrid vesicles using cancer cells.

Results and discussion

In continuation of our recent results on the temperature sensitive coiled coil peptides consisting of γ-amino acids, we hypothesized that bulky and environment sensitive fluorescent amino acids such as coumarin and NBD derived amino acids at the hydrophobic “\(α\)” and/or “\(d\)” positions may serve as suitable candidates to disrupt the coiled coil organization by changing the pH of the environment. In order to understand the compatibility and tolerance of coiled coils towards the accommodation of fluorescent amino acids at the hydrophobic positions (a or d), we adopted modified heterodimeric coiled coil engineered variant of GCN4 pLI reported by Fairman and colleagues\(^12\) (Scheme 1). The sequences of the coiled coils \(P1\) and \(P3\) are shown in Scheme 2. The hydrophobic fluorescent coumarin amino acid mutated variants of \(P2\) and \(P4\) were derived from \(P1\) and \(P3\), respectively (Scheme 2). Similarly, NBD-derived amino acid containing peptid \(P5\) was designed from \(P3\) by replacing of Ile11 with NBD amino acid (Scheme 2). The coumarin amino acid was synthesized starting from the N\(^2\)-Cbz-protected-β-keto-γ-amino ester as reported earlier.\(^17a\) While, NBD amino acid was synthesized through direct amidation of NBD-Cl with free amine side-chain of N\(^4\)-Fmoc-protected diamino propanic acid via SNAr reaction (See ESI).\(^17b\) All peptides were synthesized by solid phase method on MBHA Knorr amide resin and purified using reverse phase HPLC. We used circular dichroism (CD) to study the conformational properties of designed coiled coil peptides. The CD spectra were acquired for all individual peptides as well as an equimolar \(P1P3\), \(P1P4\), \(P2P3\), \(P2P4\), \(P3P5\) and \(P4P5\) mixture in PBS buffer at pH 7.4. The CD analysis revealed that all individual peptides were largely unfolded and displayed no secondary structure. On the other hand, the equimolar mixture of \(P1P3\), \(P1P4\), \(P2P3\), \(P2P4\), \(P3P5\) and \(P4P5\) adoted coiled-coil helical conformations (Figure S3). In order to understand the influence of pH change on the coiled coil conformation, we subjected all coiled coils to pH studies. The CD spectra were recorded at different pH and the results are shown in Figure 1. The change in the pH of the environment resulted in no change in the coiled coil conformation of control peptides \(P1P3\). Striking results were observed in the case of coiled coils composed of NBD amino acid incorporated coiled coils, \(P3P5\) and \(P4P5\). Either increase or decrease in the physiological pH leads to the transition from structured coiled coils to unfolded structures (Figure 1B and 1C).

Table 1: Thermodynamic parameters obtained from CD-thermal denaturation analysis of heterodimers

<table>
<thead>
<tr>
<th>Peptide Complex</th>
<th>(\Delta H(T_m)) (kcal mol(^{-1}))</th>
<th>(T_m) (K)</th>
<th>(\Delta G^0) at 310 K (kcal mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(P1P3)</td>
<td>-30.1810</td>
<td>336.16</td>
<td>-8.7505</td>
</tr>
<tr>
<td>(P1P4)</td>
<td>-31.2353</td>
<td>325.53</td>
<td>-7.8886</td>
</tr>
<tr>
<td>(P3P5)</td>
<td>-32.8295</td>
<td>317.26</td>
<td>-7.1640</td>
</tr>
<tr>
<td>(P4P5)</td>
<td>-33.6369</td>
<td>314.74</td>
<td>-6.8996</td>
</tr>
</tbody>
</table>
In order to understand whether the fluorescent amino acids also influence the conformational change in the coiled coils with respect to the temperature, we studied their conformational behavior using temperature dependent CD. All coiled coil peptides showed cooperative thermal unfolding upon increasing temperature from 298 K to 333 K (Figure S4). Analysis reveal that the coiled coil structure of P1P3 showed the greater stability followed by the peptides with single mutation either with coumarin or NBD at the 11d or 11d’.

Figure 2: Morphology of the coiled coil liposome hybrid vesicles and the control liposome (without coiled coils). (A) and (B) are the SEM images of drug encapsulated liposomes with and without coiled coil peptides respectively. The TEM images are shown as insets in the SEM images. (C) and (D) are representing the DLS profile of the drug encapsulated with and without coiled peptides respectively. (E) and (F) are the AFM images of coiled coil liposome composites and the control liposomes respectively. (G) Circular dichroism spectra of peptide liposome hybrid vesicles at pH 7.4 and 5.5. (H) Percentage of the drug release from the liposome at pH 5.5.

positions. Instructively, the coiled coil heterodimer, P4P5 composed of both fluorescent amino acids at 11d and 11d’ showed the considerable drop (~21 K) in their thermodynamic stability compared to the unmodified coiled coil, P1P3 (Table 1). Using temperature dependent melting data from CD spectroscopy, we calculated free energy of the coiled coils and the results are shown in Table 1. In addition all peptide showed thermo reversible folding properties, upon cooling the unfolded coiled coils regain their coiled coil signature. The

\[ \Delta G^\circ = -8.7 \text{ kcal mol}^{-1} \]

\[ \Delta G^\circ = -6.8 \text{ kcal mol}^{-1} \]

\[ \Delta G^\circ = -7.1 \text{ kcal mol}^{-1} \]

\[ \Delta G^\circ = -6.8 \text{ kcal mol}^{-1} \]

The remarkable pH sensitive signature displayed by the coiled coil heterodimer, P3P5 encouraged us to explore this coiled coil as pH sensitive trigger for the controlled release of encapsulated molecules from liposomes. In order to validate our hypothesis, we synthesized coiled coil peptide liposome hybrid vesicles by incorporating P3P5 in the bilayer of liposome. We achieved the insertion of coiled coil into the liposome by mixing it with PC/DSPE-PEG_{2000} (98:2) lipid combination before bilayer formation at the molar ratio of 10:1 (lipid: peptide) as reported earlier. These hybrid coiled coil liposome composites along with control liposomes without coiled coils were subjected to the DLS, SEM, TEM and AFM analysis to understand their vesicle signature and morphology (Figure 2). Similar to the control liposome without coiled coils, scanning electron microscopy analysis showed the homogenous vesicle like signature of peptide liposome composites. DLS studies showed mean diameter approximately 172 ± 2.34 nm with PDI value of 0.322 ± 0.005 (Figure 2C). The TEM analysis further supported the characteristic hollow-structured signature of vesicles (Figure S5). To understand whether or not the P3P5 retained its coiled coil conformation within the lipid bilayer, we subjected coiled coil liposome nanocomposites to the CD analysis. The CD analysis reveals that the P3P5 peptides retained the coiled coil signature in the liposome bilayer by displaying characteristic CD minima at 208 and 222 nm. To gain knowledge whether the P3P5 within the bilayer will undergo conformational change upon changing the pH, we adjusted the pH of the solution to 5.5. As predicted, P3P5 lost its structured coiled coil conformation upon changing the pH. Results are shown in Figure 2G. These control experiments motivated us to study the encapsulation and control release of drugs from pH sensitive coiled coil liposome nanocomposites. In order to validate acidic pH triggered enhanced drug release, we encapsulated known DNA intercalator proflavine hydrochloride into the coiled coil liposome composite. The excess drug was removed using Sephadex size exclusion chromatography. We further measured the amount of the drug encapsulated in the liposomes with and without coiled-coils. The concentration of the drug that was encapsulated by the liposome with coiled-coils was found to be 57 μM and the concentration of the drug in the liposomes without coiled-coils was found to be 36 μM. The liposomes were diluted with the buffer to achieve similar concentration of encapsulated drug for further studies. The drug loaded coiled coil liposome
composites were further subjected to the DLS, SEM and TEM analysis to understand the morphology. The DLS and other microscopic analysis suggested that there is no change in the gross morphology as compared to the control liposomes without coiled coil (Figure 2). To understand stability of drug encapsulated coiled coil liposome nanocomposites, we

Figure 3: Effect on cell proliferation on LN229 cells with (A) Direct treatment of proflavine (free drug) and treatment of encapsulated proflavine in P3P5-liposome and P1P3-liposome. (B) Treatment of peptides P3 and P5 to understand the toxicity of peptides in cancer cells.

Figure 4: Cellular internalization of (a) P3P5-liposome and (b) P1P3-liposome in LN229 cells. Time dependent internalization experiment 1hr (A), 3 hrs (B), 7 hrs (C) analyzed under confocal laser scanning microscopy. Lysosomal compartments and nuclei were stained by LysoTracker Red DND 99 and DAPI, respectively. Merged images show the colocalization of the proflavine in the acidic lysosomal compartments. (Scale : 10 µm for cell image a and 5 µm for cell image b)

Figure 5: Schematic representation of the liposomal drug delivery.

monitored their morphology over two weeks using DLS and these results suggested the remarkable stability of drug loaded coiled coil liposome over two weeks (Figure S6) without changing their gross morphology. Further, we investigated whether change in the pH enhances the release of drug from the coiled coil liposome nanocomposites, we subjected drug encapsulated both control liposome without coiled coil peptides as well as liposome with coiled coil peptides (P3P5 and P1P3) to pH 5.5. The results are shown in Figure 2H. As anticipated, the liposomes with P3P5 coiled coil peptides showed enhanced release of encapsulated drug molecules relative to the control liposome with P1P3 coiled coils as well as liposomes without coiled-coils. This data further supports that control coiled-coil P1P3 are not sensitive to the pH 5.5. These results encouraged us to validate the viabilities of these liposomes for the specific delivery to the cancer cells.

To verify the potential effect of the peptide liposome hybrid vesicles on cell proliferation, we subjected them to MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay using glioblastoma cells LN229 as a proof of concept. The cells were treated with various concentrations of free proflavine and proflavine encapsulated coiled-coil liposome. As shown in Figure 3, at the same concentrations, the free drug displayed higher cytotoxicity (IC\textsubscript{50} value 0.083 µM) compared to the proflavine encapsulated P3P5-liposome hybrid vesicles (IC\textsubscript{50} value 2.58 µM). This can be attributed due to the controlled release of the drug molecules from the hybrid vesicles. However, at higher concentrations, as expected, comparable cytotoxicity has been observed from the liposomes. In contrast, no or less toxicity has been observed with drug encapsulated liposomes containing control P1P3 coiled-coils. These results further suggest that in comparison with P3P5-liposomes, there is no or very slow release of drugs from the control P1P3-liposomes. Further, we evaluated whether the individual coiled coil peptides are cytotoxic to the cells. Experimental results suggested that both individual peptides P3 and P5 displayed 80% cell viability up to 25 µM concentration in 48 hrs.

To realize the cellular uptake and controlled release of proflavine from the encapsulated coiled coil liposome hybrid vesicles, we further examined glioblastoma LN229 cells in time dependent manner using confocal laser scanning microscopy (CLSM). The green fluorescent property of the drug was exploited in tracing the localization of drug in the cells. After 1 hr a green fluorescence observed in the cells indicating the successful cellular uptake of the drug encapsulated liposomes. With increasing of time, significant enhancement of
fluorescence signal suggesting the continuous accumulation of drug from the coiled coil hybrid liposomes. The images are shown in Figure 4a. In a sharp contrast, low internalization and poor release of proflavine from the control liposomes without coiled coils (See ESI Figure S12) and liposomes with P1P3 peptides (Figure 4b) was observed. In order to localize the subcellular distribution of internalized liposome encapsulated drug, the cells were stained with DAPI and LysoTracker Red. The overlap of red and green fluorescence suggests the localization of drug mainly in the acidic lysosomes. The amount of localization of the drug was further calculated using Pearson's correlation coefficient and Manders coefficients by CLSM.19 As predicted, the acidic pH of the lysosome induced the unfolding of coiled coil, which resulted in the disorder of liposomal bilayer and helped in releasing the drugs from vesicles. The schematic representation of the acidic pH triggered release of encapsulated drugs from the coiled coil liposome hybrid vesicle is shown in Figure 5. Overall, these results suggested the enhanced and control delivery of drugs from the pH sensitive, non-cytotoxic coiled coil liposome nanocomposites, which opens new possibilities in the design of pH sensitive peptide hybrid vesicles for the delivery of biologically important molecules.

Conclusion

We have demonstrated the design and utilization of coiled coil peptides as pH sensitive triggers for the enhanced release of entrapped drug molecules from the liposomes. As hypothesized introduction of environment sensitive fluorescent amino acids as guests into the host coiled coil peptides lead to the pH sensitive coiled coils without much deviation from the overall conformation at neutral conditions. The pH mediated transition from the structured coiled coil to unstructured peptides was further exploited as triggers for the release of encapsulated drug molecules from the liposomes. The time course experiments revealed that the coiled coil liposome nanocomposites were stable up to two weeks without any change in their gross morphology at physiological pH. In contrast to the control liposomes, the coiled coil liposomes displayed enhanced internalization and colocalization in the low acidic lysosomal compartments of cancer cells. The pH sensitive enhanced cell internalization property of liposome coiled coil nanocomposites reported here may guide to design promising smart biomaterials which can be used as tunable drug delivery systems for the cancer therapy.

We thank Department of Science and Technology, Govt. of India for financial support. RMR thanks IISER Pune and R.M thankful to CSIR, Govt. of India for research fellowship.

Experimental

Materials: All N-Fmoc protected amino acids, MBHA knorr amide resin, O-(benzotriazol-1-yl)-N, N', N''-tetramethyluronium hexafluorophosphate (HBTU), dimethyl formamide (DMF), N-methyl-2-pyrrolidone (NMP), diisopropylethylamine (DIPEA), piperidine, trifluoroacetic acid (TFA), triisopropyl silane (TIPS), α-phosphatidylcholine (PC), monobasic potassium and dibasic sodium phosphate, sephadex G-25 and 3,6-Diaminoacridone hydrochloride (Proflavine), 4-Chloro-7-nitrobenzofuran (NBD-Cl), IBC-Cl, Methanesulphonic acid, 3-Methoxyphenyl, 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) [DSPE-PEG(2000)] were procured from the commercial sources. All biophysical characterizations were performed in phosphate-buffered saline (PBS: 10 mM phosphate, 150 mM NaCl at pH 7.4 and 10 mM acetate buffer at pH 5.5). In case of pH dependent studies, acidic pH was achieved by adding dilute acetic acid and basic pH was achieved by adding solid K2CO3. The final concentrations of all peptides were prepared from their respective stock solution as needed.

Peptide Synthesis: The N-acetylated peptides, P1-P5, were synthesized on a MBHA Knorr amide resin at 0.1 mmol scale by manual solid phase synthesis using standard Fmoc chemistry. The coupling reactions were carried out using HBTU/HOBt coupling agents. The Fmoc deprotection was carried out using 20% piperidine in DMF. N-Acetylation of the peptides was carried out using acetic anhydride/pyridine (1:9). Peptide cleavage from the resin was achieved by treatment of the resin with a cocktail mixture of trifluoroacetic acid (TFA)/triisopropylsilane/water (90:5:5) for 2 hrs. Then the resin was filtered with additional TFA (5 mL) and the combined extracts were concentrated under vacuum. The crude peptide was then precipitated in cold diethyl ether (30 mL) and isolated by centrifugation. The precipitate was redissolved in 5 mL of a 1:1 mixture of acetonitrile/water and then lyophilized to give a fine white solid. The crude peptide was subjected to purification using reverse phase HPLC using C18 column. The mass of the pure peptides were confirmed using MALDI-TOF/TOF.

Circular Dichroism (CD) Spectroscopy: Temperature and pH dependent circular dichroism spectra were recorded using a JASCO J-815 spectropolarimeter fitted with a peltier temperature controller. CD spectra were measured at 30 μM total peptide concentration (15 μM each) in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) at 20 °C in 2 mm quartz cuvettes at 50 nm/min scanning speed. Thermal-denaturation experiments were performed by heating from 20 to 60 °C at a rate of 1 °C/min. The CD signal at 222 nm was recorded at 5 °C intervals. For the pH dependent circular dichroism, acidic pH was achieved by adding dil. AcOH and basic pH was achieved by addition of K2CO3 solution. Each experiment was performed in triplicate.

Preparation of Peptide Liposome Hybrid Vesicle:

Peptide liposome hybrid vesicles were prepared as reported earlier. Briefly, egg yolk L-α-phosphatidylcholine (EYPC, 15.37 mg, 20 μmol) and DSPE-PEG2000 (0.14 mg, 0.5μmol) were
dissolved in 1 mL DCM. Then, 200 µL solution of \textbf{P3/P5} (1:1, 15 µmol) was prepared by diluting their stock solution in MeOH. Both the organic solutions (DCM and MeOH) were mixed together in small 25 mL round bottom flask. A thin and uniform lipid-peptide bilayer was formed after slow evaporation of organic solvents under vacuum. The bilayer film obtained after complete evaporation of organic solvents, was hydrated with 1 mL solution of 0.1 mM Proflavine hydrochloride solution in PBS buffer (10 mM phosphate, 150 mM NaCl, and pH 7.4). Hydration process was carried out for 1 hour with continuous agitation at room temperature and occasional sonication. Then, the extra-vesicular proflavine salt and the peptides were removed by size exclusion chromatographic separation using Sephadex G-25 column. Liposome containing aliquots were collected and small unilamellar liposomes were obtained after 20 times extrusion through 100 nm polycarbonate membrane using mini-extruder. The unilamellar liposomes were again subjected to Sephadex G-25 column to remove free drug liberated in the process of extrusion. The size and morphology of the liposomes were confirmed using FE-SEM and TEM. The liposomes were stored at 8 °C for the further use. Similar protocol control was used for the liposome without peptide.

**Field Emission-Scanning Electron Microscopy (FE-SEM):**
Samples were prepared by diluting 50 µL of liposome solution to 1 mL by the addition of double distilled water. Then, 5 µL of the solution was drop cast on silicon wafer. Samples were allowed to dry at room temperature and then coated with gold. Scanning electron microscopic imaging was performed using electron microscope operating at 30 kV.

**Atomic Force Microscopy (AFM):**
Peptide liposome hybrid vesicle solution (50 µL) was diluted to 1 mL by addition of double distilled water. Then, 5 µL of the solution was drop casted on a mica foil. The morphology and size of the hybrid vesicles was measured after complete drying of the sample.

**Transmission Electron Microscopy (TEM):**
TEM samples were prepared by diluting 50 µL of the liposome solution to 1 mL by addition of double distilled water. Then, 5 µL of the solution was drop casted on the copper grid, dried at room temperature, and the image was taken. Same procedure was followed for the control liposome sample.

**Size Distribution Analysis of Hybrid Nanoparticles by DLS:**
Mean diameter of liposomes was measured by dynamic light scattering (DLS) experiment using 90° scattering angle. Samples were prepared by diluting 50 µL liposome solution by the addition of 1 mL double distilled water.

**Procedure for pH Triggered Percentage of Drug Release:**
Lipid-peptide hybrid liposome (200 µL) loaded with proflavine hydrochloride was sealed in dialysis membrane having molecular weight cut off 500 Da. The dialysis bag was suspended in agitating PBS buffer (3 mL, 10 mM phosphate, 150 mM NaCl, and pH 7.4) and acetate buffer (3 mL, 10 mM acetate buffer at pH 5.5), at the 37°C, separately. Control studies were also performed at identical conditions using liposome without peptides. The aliquot 200 µL from the suspension medium was timely collected and diluted to 400 µL with respective buffer solution. Then quantification of released proflavine was carried out using UV/Vis spectrometer at λ\textsubscript{max}=444 nm. The percentage of the drug release was calculated from corresponding calibration curve for the Proflavine hydrochloride in the buffer solution. All experiments were repeated in triplicates.

**Procedure for MTT Assay in LN229 Cell Line:**
LN229 cells were seeded in 96 well plate at a density of 5000 cells per well in 200 µL of DMEM medium supplemented with 5% FBS and allowed to attain morphology overnight at 37 °C and 5% CO₂ incubator. Cells were treated with different concentration of free drug (5, 10, 15, 20 & 25 µM) and peptide liposome hybrid encapsulated with equivalent amount of drug for 48 h. After 48 h, 20 µL of MTT (5 µg/µL) reagent was added to the cells and allowed to form formazan crystals for 4h at 37 °C and 5 % CO₂. After 4h, the media was discarded without disturbing the formazan crystals. Crystals formed by the viable cells were dissolved in DMSO and quantified by measuring the O.D. at 570 nm with Elisa reader. Percent survival of cells upon treatment with drug only and drug encapsulated with liposome was calculated by comparing the amount of formazan crystal formed with untreated cells.

**Procedure for the Cell Internalization in LN229 Cell Line:**
Cell internalization was performed using 2 x 10⁴ LN229 cells, which were seeded on coverslip in 24 well plate and incubated at 37 °C and 5% CO₂ to allow them to attain their morphology. Cells were treated with 0.2 µM of free proflavine as well as peptide liposome encapsulated proflavine for 1h, 3h and 7h. Cells were also treated with Lyso Tracker DND 99 for about 30 minutes. After specific time intervals, cells were washed thrice with PBS and fixed for about 15 minutes with 4% PFA at room temperature. Cells were again washed thrice with PBS for 5 minutes each. To stain nucleus, cells were incubated with 2 mg/ml Hoechst for 15 minutes at room temperature. To remove unbound Hoechst, cells were washed three times with PBS and mounted on glass slide with mounting medium. Imaging was performed using confocal microscope. Lyso Tracker, Hoechst and proflavine were excited at 577 nm, 405 nm and 444 nm respectively.

**Notes and references**


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