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Stimuli-Responsive Anticancer Drug Delivery System with Inherent Antibacterial Activities

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We describe a novel class of stimuli-sensitive synthetic lipids, which possesses several favorable biophysical properties of phospholipids. The sulfonium-based lipid was successfully disassembled by glutathione to release the encapsulated drug molecules in a controlled manner. The cationic lipid also showed lower cytotoxicity against mammalian cells and displayed moderate antibacterial activities.

The delivery of drug molecules to the specific cells or tissues through the blood circulation system is one of the major impediments in pharmaceutical research.[1-3] Advanced drug delivery strategies including the lipid-based nanoparticles are considered as a promising approach to effectively transport a wide range of therapeutic agents including the hydrophobic drug molecules to the targeted cells or tissues with apposite therapeutic doses.[1-5] The biodegradability, non-immunogenicity, and others are the unique felicitous properties of the lipid-based drug delivery system.[6, 7] The growing number of FDA approved lipid-based formulations, including Doxil®, Ambisome®, and DepoDur™ provide evidence of their enormous potential as a capable drug carrier.[8] The structure of the natural phospholipids has been extensively modified to optimize its self-assembly, serum-stability, circulation time, targeted delivery, circumventing side effects, and others. The modification of lipid head-group through the installation of phosphate bioisosteres, polyethylenes, cationic moieties, cell-specific ligands, and others have been exploited to augment its stability, circulation times, cell-specific drug delivery ability.[4, 6-11]
Currently, the stimuli-responsive drug delivery strategy is considered as one of the practical approaches in terms of drug efficacy, especially for cancer treatment.[4, 12, 13] Significant changes in internal physiological conditions, including the pH, redox potential, protein/enzyme levels, and others of the tumour microenvironment, have been used as stimuli for chemotherapeutic drug delivery applications. These stimuli cause a significant change in the chemical structure of the drug carriers due to the cleavage of specific functional groups or changes in physicochemical properties, which instigate the release of encapsulated drug molecules in-or-around the diseased cells.[4, 12] The glutathione (GSH)-sensitive lipids may provide a practical approach to develop potential drug carriers. Using the thiol-disulfide exchange reaction between GSH and disulfide bond of the carrier and the irregularity of GSH concentration between normal cells and cancer cells, a large number of complex polymeric materials have been developed as drug carriers.[4, 8, 13] Unfortunately, GSH sensitive vesicle formulation has not been well explored. Recently, the GSH-sensitive disulfide containing phosphatidylcholines (SS-PCs) has been demonstrated.[14] However, this redox-sensitive disulfide-based nanocarrier showed a quick response to S-S bond cleavage in the cellular microenvironment. This high reactivity of GSH towards the S-S bond limits its intracellular uptake and increases the risk of cytotoxic effect.[14, 15]
To curtail the problems related to the high reactivity and low cellular uptake abilities of the disulfide-based liposomal drug delivery system, we introduced the sulfonium-based lipids. The sulfonium moieties are naturally occurring and present in several therapeutic agents including, bleomycin, and adenosylmethionine. It is reported that the sulfonium containing compounds are less toxic to the mammalian cells in comparison with the other onium analogous.[16] The higher concentration of GSH is only known to cleave the sulfonium moieties in a controlled manner.[15] We postulate that the presence of the sulfonium moiety could improve the cellular uptake efficiency and drug release profile of these lipids in comparison to disulfide-containing lipids.
Herein, we synthesized a series of novel sulfonium-based lipids, which showed a wide range of favourable biophysical properties such as the formation of aqueous soluble spherical aggregates, high phase transition temperature, stimuli-responsive system, and others. Additionally, the potent lipid has lower cytotoxicity, higher efficiency to encapsulate, and release hydrophobic drug,

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doxorubicin (Dox) to the cancer cells. The cellular uptake efficiency in the absence and presence of GSH inhibitor demonstrated that the intracellular GSH level acts as a stimulus to release the drug molecules in cancer cells. Additional studies revealed that the sulfonium-based lipid itself has moderate antibacterial activity against both gram-positive and gram-negative bacteria.

The novelty and recent applications of the GSH-responsive drug delivery approach motivated us to design sulfonium-based lipids (Fig. 1A). These modular lipids were synthesized using 1,3,5-tris(bromomethyl)benzene as the core unit. The mono-modification with azide and trimethylamine resulted in the formation of compounds **2** and **3**, respectively. The nucleophilic addition of aliphatic thiols with compounds **2** and **3** yielded compounds **4** and **5**, respectively. The azide-alkyne click reaction of compound **4** with propynesulfonic acid produced compound **6**. Finally, the S-methylation of compounds **4**, **5,** and **6** yielded the target compounds **SL1**, **SL2,** and **SL3**, respectively, with good yields (Scheme S1).[15] For all these SL-lipids, the long alkyl chains (tail lengths of 12 and 16) were associated with sulfonium moieties. The presence of azide group provides access to install various headgroups. We hypothesize that the nature and inherent properties of these synthetic sulfonium lipids would be quite different from that of natural glycerophospholipids. Hence, it is vital to explore its physicochemical properties.

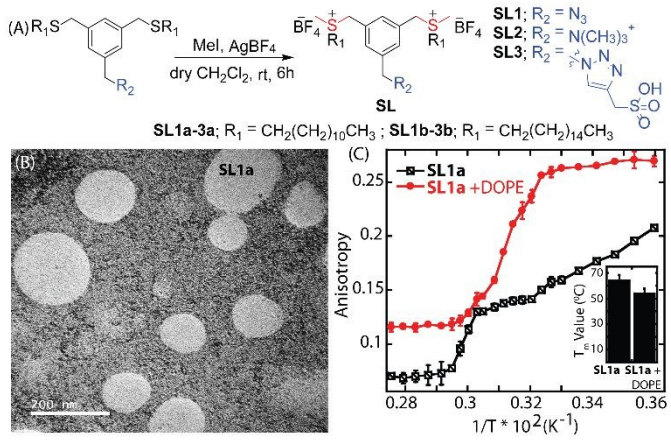

Fig. 1. Synthetic routes to the sulfonium-based lipids (A). The TEM image of the soluble aggregates generated from the 100% **SL1a** lipid (B). The steady-state fluorescence anisotropy measurement of 1,6-diphenyl-1,3,5-hexatriene of the lipids within the range of 5–90 °C. The Inset shows the T_m values of the lipids (C).

The hydration of the dried film of the **SL1a** lipid ensued in the formation of colloidal aggregates. The analysis of transmission electron microscopic (TEM) images revealed the formation of spherical aggregates of the **SL1a** lipids (Fig. 1B).[6, 7] The TEM analysis also revealed that the mixture of **SL1a** and dioleoylphosphatidylethanolamine (DOPE) lipids (1:4) form excellent vesicles (Fig. S1), suggesting the competence of **SL1a** lipid in forming vesicles with other phospholipids, which could be beneficial in constructing size-tunable vesicles for specific uses like bio-imaging and drug delivery. The transmission electron microscopy-energy dispersive X-ray spectrometry (TEM-EDX) elemental mappings of sulfur provided direct evidence of the self-assembled structure of **SL1a** lipid (Fig. S1). However, the other synthetic lipids were less efficient in the formation of any soluble aggregates, which could be due to their higher hydration energies. Hence, further biophysical characterizations were performed only with the **SL1a** lipid. The dynamic light scattering (DLS) measurement revealed that the hydrodynamic diameters (d_H) of the vesicles were within 200-450 nm, and the pH of the solution has a negligible effect on its d_H values (Fig. S2).[6, 7] The zeta-potential measurement at various pH values revealed that the vesicles form positive surface potential, which could be due to the presence of sulfonium moieties (Fig. S3). The decrease in surface potential values with the increase in pH suggest that the reduction of sulfonium positive charge could be due to the increase in the solvation number of the negatively charged hydroxyl group.

The temperature-dependent steady-state fluorescence anisotropy measurements revealed that the phase transition temperature (T_m) of **SL1a** lipid was 64 °C (Fig. 1C).[6, 7] The higher T_m values demonstrate higher thermal stability of the vesicles. Whereas, the T_m value of co-liposome (**SL1a**: DOPE of 1:4) was 54 °C, which is much higher than phospholipid, DOPE (T_m = -16 °C), suggesting that the presence of sulfonium moieties plays a crucial role in their molecular arrangements. The higher T_m value of the **SL1a** vesicle and co-liposome than normal human body temperature (37 °C) also suggests their better drug retention ability in comparison with only DOPE, which indicates their prospective application in thermally triggered drug delivery systems. It is important to mention that the increase in kinetic energy of the vesicles with the increase in temperature could induce the rearrangement of their aggregation pattern prior to or post to their phase transition.

The presence of sulfonium groups encouraged us to investigate whether the vesicles can be deformed in a controlled manner in the presence of stimuli like GSH and others. To confirm the dealkylation of **SL1a** lipid, we performed the HPLC analysis in the absence and presence of GSH (10 mM) in PBS (pH 7.4) at 37 °C (Fig. 2A). The time-dependent HPLC analysis revealed that the formation of mono- and di-dealkylated intermediates start after 36 and 72 h of incubation (Fig. 2A and B). The dealkylations of **SL1a** lipid were scrutinized by mass spectrometry (Fig. S4 and S5). The control experiment showed that the **SL1a** lipid is very much stable in the absence of GSH under similar experimental conditions (Fig. S6). The TEM and TEM-EDX analysis of the **SL1a** lipid in the presence of GSH also showed a significant change in their aggregation arrangements, indicating its instability under the reduced environment (Fig. 2C and S7).

This stimuli-responsive dealkylation-mediated deformation of vesicles of **SL1a** lipid prompted us to investigate its drug encapsulation and release profiles (Fig. S9). The **SL1a** lipid showed adequate loading aptitude (33% at pH 7.4) for hydrophobic anticancer drug Doxorubicin (Dox). This moderate drug loading capacity of **SL1a** lipid suggests that the positively charged hydrophobic Dox (pK_a = 8.4) molecules could be entrapped within the hydrophobic core of the positively charged lipid assembly. Fluorescence studies revealed that 57% and 88% of Dox was released after 60 h in the absence and presence of GSH, respectively at 37°C (Fig. 2D).[6, 7] The release-profile also revealed a drastic difference after 36 h, suggesting the possibility of the destabilization of **SL1a** lipid assembly in the

presence of GSH. This time-dependent release profile is in accordance with the HPLC data. This controlled Dox release profile of **SL1a** lipid is advantageous for its further biological applications, including a reduction in side effects due to nonspecific delivery and enhancement of the activity the Dox and others.

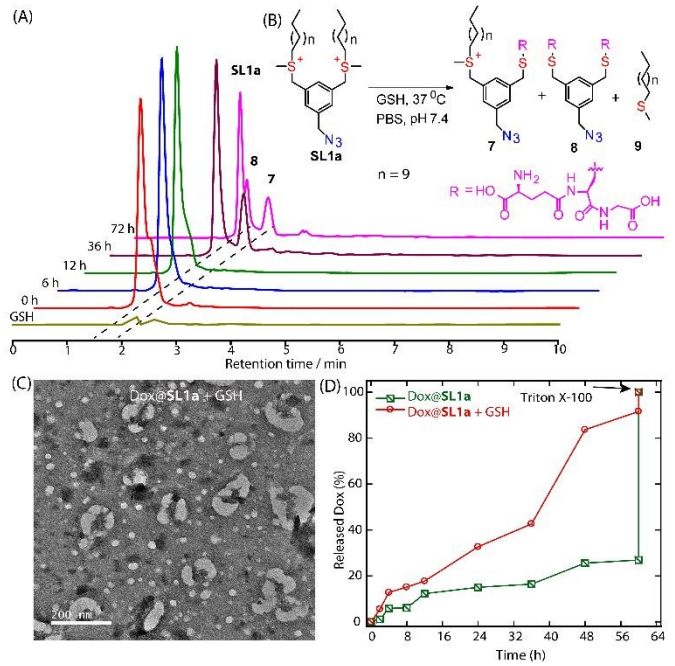

Fig.2. The HPLC traces for the dealkylation of lipid **SL1a** in the presence of GSH (10 mM) at different time intervals (A). Schematic representation of the dealkylation strategy (B). The dealkylation of compound **SL1a** (1 mmol) in the presence of GSH (10 mmol) in PBS (pH = 7.4) at 37 °C. The TEM image of the **SL1a** lipid in the presence of GSH (10 mmol) after 36 h of incubation (C). The Dox release profile of the **SL1a** lipid at pH7.4 in the absence and presence of GSH (10 mM).

The MTT-based viability assay showed that the **SL1a** lipid had lower cytotoxic effect against MDA-MB-231 (human triple-negative breast cancer; IC_{50} = 125 µM) and BJ (human foreskin fibroblasts; IC_{50} = 166 µM) cell lines, suggesting its relevance in drug delivery applications (Fig. S10A and S10C).[2, 7] The MTT assay in the presence of free Dox and Dox encapsulated **SL1a** lipid (Dox@**SL1a**) revealed that the IC_{50} values were 0.49 and 0.36 µM (with respect to effective Dox concentration), respectively in MDA-MB-231 cell line, which support the Dox encapsulation and release efficiency of **SL1a** lipid (Fig. S10B). The IC_{50} value of Dox@**SL1a** treated BJ cells was 1.47 µM (with respect to effective Dox concentration; Fig. S10D). The higher concentration of GSH levels in MDA-MB-231 cells attributed to the cleavage of sulfonium lipids, resulting in much lower IC_{50} value of the Dox@**SL1a**.[17] The final concentration of Dox was kept same for both the MTT assay in the presence of free Dox and Dox@**SL1a**. The time-dependent MTT assay showed that the rate of cell death was faster for Dox@**SL1a**, indicating its superior uptake and release of Dox molecules, which is one of the rudiments of a potential drug carrier (Fig. 3A). To further investigate that the GSH promotes the Dox release efficiency from Dox@**SL1a** under the cellular environment, the MTT assay was performed in the absence and presence of GSH inhibitor, buthionine sulfoximine (BSO).[15] The result showed that the cell viability increased in the BSO treated cells, which could be due to the inhibition of GSH activity (Fig. 3B and S11).[15]

The cellular uptake efficiency of **SL1a** lipid was further investigated using a confocal laser scanning microscope (CLSM).[6, 7] The microscopic images of the MDA-MB-231 cells revealed a higher Dox delivery efficiency of the **SL1a** lipid in comparison with the lipid **4a** (Fig. 3C, S12-14). The long alkyl chains of the neutral lipid **4a** were associated with only thioether moieties. To exclude the probability of fluorescence signal originated due to the adherence of the Dox@**SL1a** or Dox@**4a** to the cell surface, the flow cytometry analysis was performed using the inherent fluorescence signal of Dox molecules (FL2-H channel). The result showed that the Dox@**SL1a** has a higher cellular uptake efficacy in comparison with that of Dox@**4a in** MDA-MB-231 cells (Fig. S15), which could be due to the superior electrostatic interaction of the positively charged sulfonium moieties with the negatively charged phosphatidylserine (PS) lipid present in higher abundance on the outer surface of the cancer cells.[18] This directed electrostatic interaction could also help the cationic sulfonium lipids to reduce the off-target side effects of the Dox itself. The higher cellular uptake of free Dox could be due to its diffusion–mediated delivery to the cells, whereas liposome-mediated Dox delivery generally follows the endocytosis process (Fig. S15).[2,7]

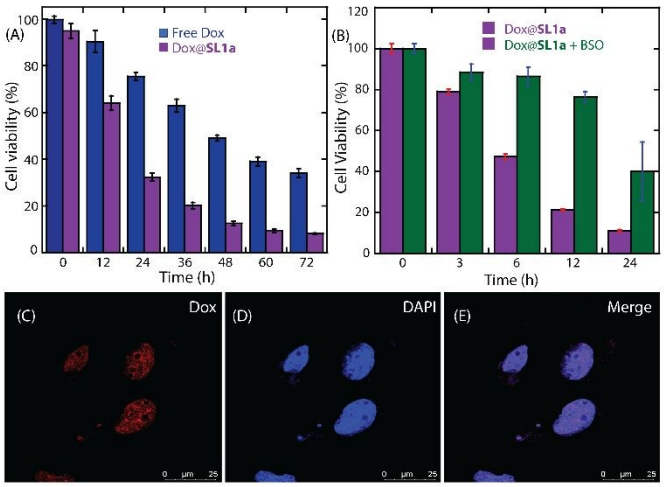

Fig.3. Time-dependent viabilities of MDA-MB-231 cells in the presence of free Dox and Dox@**SL1a** at 0.36 and 0.49 µM, respectively (A). The effective concentration of Dox in Dox@**SL1a** was 0.49 µM (calculated using the loading capacity). Viabilities of the Dox@**SL1a** (0.49 µM) treated MDA-MB-231 cells in the absence and presence of glutathione inhibitor, BSO (1 mM) (B). CLSM images of the MDA-MB-231 cells treated with Dox@**SL1a** (0.49 µM) for 8 h. Red channel (C), blue channel (D), merge of red and blue channels (E) illustrate the Dox delivery. Scale bar 25 µm.

The presence of sulfonium moieties and lower cytotoxicity against mammalian cell lines endorsed us to investigate the antibacterial activities of **SL1a** lipid.[16] The antibacterial activity of the **SL1a** lipid was tested against both gram-negative (*E. coli*; MTCC-1687) and gram-positive (*S. aureus*; MTCC-96) bacteria and the minimum inhibitory concentration (MIC) values were 32 ± 2 and 14 ± 1 µM, respectively (Fig. 4A, S16 and Table S1). For further investigation, the antibacterial activities were also performed in the presence of free Dox and Dox@**SL1a**.

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Interestingly, the free Dox has much lower antibacterial activities (MIC values >72 and 36 μM for *E. coli* and *S. aureus*, respectively.[19] Whereas, the MIC values were 0.19 and 0.10 μM for *E. coli* and *S. aureus*, respectively (with respect to effective Dox concentration; Table S1), in the presence of Dox@**SL1a**. The field emission scanning electron microscope (FESEM) analysis showed a noticeable loss of cell shape and integrity in the presence of **SL1a** and **Dox@SL1a** (Fig. S17 and 4B). Overall, the antimicrobial studies revealed that the ammonium-free drug carrier itself possesses antibacterial activities. Recent studies revealed that the peptidoglycan of bacterial cell wall promotes the invasiveness of breast cancer cells by upregulating the Toll-like receptor 2.[20] Gram-negative bacteria like *E. coli* and *B. fragilis* are also known to produce colorectal cancer-promoting toxin.[21] Therefore, the inherent antibacterial activity of the sulfonium lipid, along with its anticancer drug delivery efficacy, represents a highly efficient stimuli-responsive delivery system.

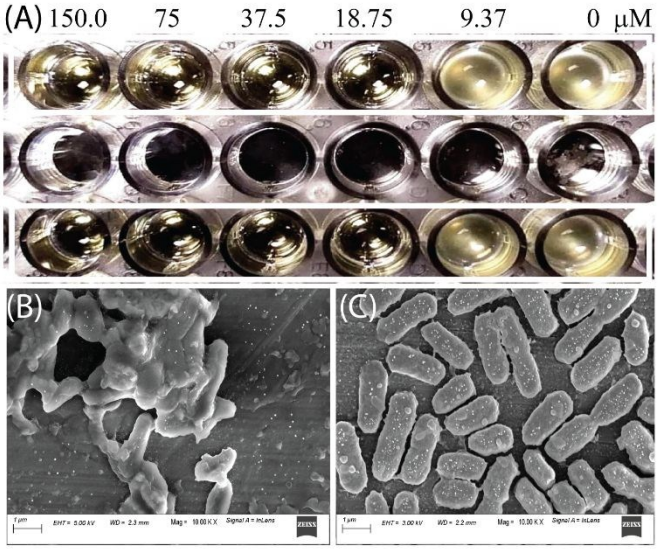

Fig. 4. The 96 well plate bacterial assay of Dox@**SL1a** (first and third row; with respect to **SL1a** concentration) against *E. coli* (MTCC-1687*S*). The clear wells specify inhibition of bacterial growth, while the cloudy wells indicate unconstrained bacterial growth. FESEM images of *E. coli* (MTCC-1687*S*) treated with Dox@**SL1a** (B) and only Dox (C).

Conclusions

The sulfonium-based modular lipids were successfully synthesized under mild reaction conditions. The potent cationic lipid self-assembled in the aqueous medium to form soluble spherical aggregates, which showed high phase transition temperature, GSH-responsive cleavage, and other favorable biophysical properties of drug delivery agents. The sulfonium lipid displayed successful encapsulation and delivery of the hydrophobic anticancer drug, Dox to the MDA-MB-231 cells. Interestingly, the sulfonium lipid itself possesses antimicrobial activities against both gram-positive and gram-negative bacteria. Hence, the sulfonium lipid could be a potential alternative to the natural phospholipids, which can fight against bacterial infection and can be cleaved in a controlled fashion under the reductive tumour environment to release the encapsulated drug molecules.

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Conflicts of interest

There are no conflicts to declare.

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