Design of photocleavable lipids and their application in liposomal "uncorking"†

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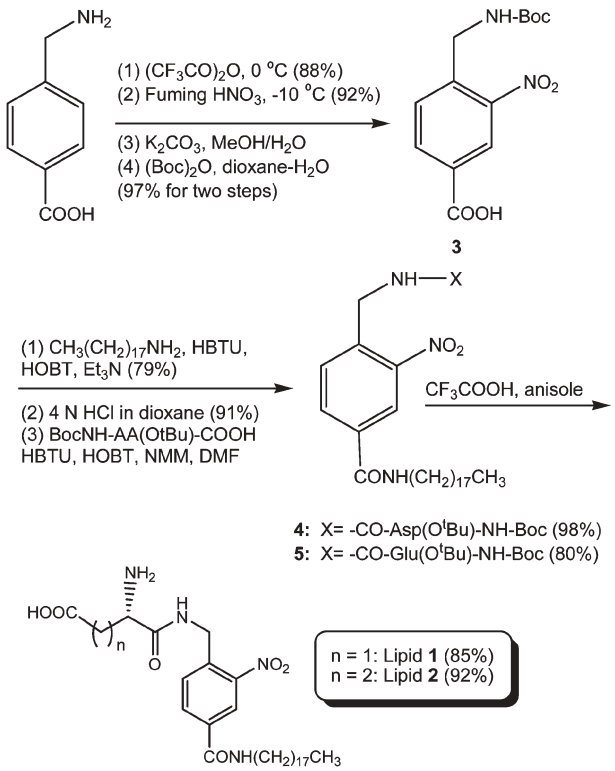
The design of *o*-nitrobenzyl containing photocleavable lipid–amino acid conjugates, and their application in liposomal uncorking are described.

The drug design/discovery endeavour of biomedical research often produces compounds that are not easily targetable to the desired site in the human body to exhibit their effects. Aside from the problems associated with solubility, biodegradation, membrane permeability, and cytotoxicity of putative drugs, their target specific "delivery" has been one of the major challenges of pharmaceutical research. Various drug carriers (*e.g.*, liposomes, polymers, micro-spheres, antibody–drug conjugates) have been developed to alter the bio-distribution and pharmacokinetic properties of drug molecules. Among such carriers, liposomes offer several advantages as clinical drug delivery vehicles, and at present, there are 13 liposome-mediated drug delivery systems approved for the treatment of a variety of human diseases (*e.g.*, breast cancer, ovarian cancer, meningitis, fungal infections, leukaemia, *etc.*).[1] In addition, the liposome mediated delivery of about 30 other small molecule drugs, DNA fragments, and diagnostic compounds are currently at different stages of clinical trials.[2]

In liposome mediated target-specific drug delivery systems, three features need to be taken into consideration: (i) appropriate coating of liposomes to circumvent their clearance by the reticuloendothelial system (RES),[3] (ii) attachment of the target-specific recognition moiety (*e.g.*, antibodies, receptor agonist/antagonist), and (iii) incorporation of the triggering mechanism.[1] Attachment of receptor-specific ligands ensures their adhesions to target cell surfaces.[1,4] Although the surface-attached liposomes can be passively internalized inside the cells and release their contents, the overall process is usually slow, unless they are subjected to external triggers. Such triggering agents include change in pH,[5] mechanical stress,[6] metal ions,[7] temperature,[8] light[9] and enzymes.[10] We recently formulated liposomes with collagen mimetic triple helical peptides, and demonstrated the trigger release of their contents by a matrix metalloproteinase, MMP-9.[11] Since MMP-9 is overexpressed in a variety of cancerous tissues,[12] the overall methodology is likely to find applications in drug delivery for suppression/attenuation of invasion and metastasis. However, the above approach has the limitation of uncorking liposomes at cancerous tissues where MMP-9 is *not* overexpressed. In pursuit of developing a more general "triggered" release methodology, we noted that *o*-nitrobenzyl substituted compounds are easily cleaved by near-UV radiation,[13] and they can be derivatized to function as photocleavable lipids. There are a few reports of the design of photocleavable lipids,[9,13] but their syntheses are rather elaborate and often time consuming. Toward this end, we developed a synthetic scheme for conjugating a C_{18}-amine and selected negatively-charged polar amino acids *via* the *o*-nitrobenzyl group as a spacer (lipids **1** and **2**, Scheme 1).

The overall synthesis was accomplished *via* four easy steps: (i) selective nitration at the *o*-position of the aminomethyl group of *p*-aminomethyl benzoic acid, (ii) conjugation of stearylamine at the carboxyl group of compound **3**, (iii) removal of the amine protecting group and attachment of the selected amino acids *via* the α-carboxyl group, (iv) final removal of the protecting groups. A detailed account of the syntheses are given in the ESI.†

Based on the literature precedent,[13] it was anticipated that the *o*-nitrobenzyl group of the lipids **1** and **2** would be cleaved upon irradiation by UV/visible light in the 320–400 nm region. The



Scheme 1 The structures of the lipids incorporating the *o*-nitrobenzyl group and their syntheses are shown.

† Electronic supplementary information (ESI) available: experimental details for the synthesis of lipids **1** and **2**; liposome formation and release conditions. See http://www.rsc.org/suppdata/cc/b5/b503423j/

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photocleavage of the *o*-nitrobenzyl group proceeds *via* abstraction of a benzylic hydrogen by the photo-activated nitro group. This is followed by an electron-redistribution to form an aci-nitro form, which finally rearranges to form the *o*-nitroso benzaldehyde product.[13,14] It is known that the precursor–nitro conjugates exhibit absorption maxima in the range of 250–270 nm,[13,14] the intermediate and final nitroso-derivatives are characterized by the red shift in the corresponding aromatic absorption band by 50–80 nm.[14,15] Hence, the time course of the overall cleavage process of the amphiphilic lipid–amino acid conjugates could be easily probed spectrophotometrically. Since lipids **1** and **2** exhibited similar spectral features, the results are discussed only for lipid **1**. Fig. 1 shows the time dependent spectral changes upon irradiation of an ethanolic solution of lipid **1** at 365 nm.

The spectral data of Fig. 1 indicate that the irradiated *o*-nitrobenzyl group of lipid **1** shows a pronounced absorption peak at 247 nm, with a broad shoulder at 300 nm, and a minor shoulder at 220 nm. As the time of irradiation increases, the intensities of all these peaks increase. However, the shoulder peak of the original (uncleaved) lipid at 300 nm is split into two peaks with absorption maxima at 290 and 315 nm respectively. Of these peaks, the latter is characterized by the formation of a "nitroso" derivative of the cleaved product.[14] Since the overall spectral changes conformed to clean isosbestic points at 218, 260, and 385 nm, it implied that there were no spectrally distinct intermediates during the course of the overall cleavage process. However, to further probe whether some kinetically significant (albeit spectroscopically undetectable) intermediate was produced during the course of the photocleavage reaction, we analyzed the time slice of the absorption changes at 315 nm. As shown in the inset of Fig. 1, the kinetic profile was best fitted by a single exponential rate equation, with a rate constant of 0.43 min^{-1}, suggesting that the overall cleavage reaction indeed involved a single step. This rate is comparable to reported cleavage rates for the *o*-nitrobenzyl group under similar irradiation conditions.[15]

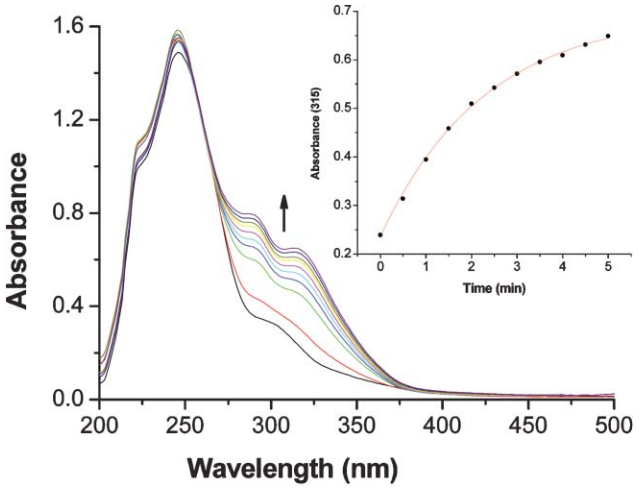


Fig. 1 Time dependent spectral changes upon irradiation of lipid **1** at 365 nm. The spectra were recorded during 5 minutes of irradiation in 30 s intervals. The inset shows the time slice of the spectral changes at 315 nm. The solid smooth line is the best fit of the data for a single exponential rate equation, with a rate constant of 0.43 min^{-1}.

Based on literature reports,[13,14] the structures of the photolysis products for lipid **1** are shown in Scheme 2.

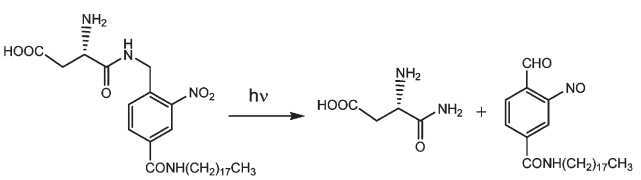
The liposomes were prepared with 1,2-distearoyl-glycero-3-phosphocholine (DSPC, 95% by weight) and 5% of the photocleavable lipid **1** in 50 mM HEPES buffer (pH = 7.0). The liposomes were characterized by transmission electron microscopy (see ESI†) and the average size of the liposomes was found to be 60–70 nm. A self-quenching hydrophilic dye, 6-carboxyfluorescein, was encapsulated in the liposomes.[16] The rate of content release will depend on the structures of the encapsulated molecules. To facilitate the release, a hydrophilic dye was selected for these studies. The excitation and emission maxima of 6-carboxyfluorescein were determined to be 495 and 518 nm, respectively (see ESI†). Due to the self quenching effect of the above fluorophore at high concentration[17] (the condition which prevails in the lumen of the liposomes due to the local concentration effect), the release of the dye from liposomes (upon uncorking) was expected to proceed in concomitance with the increase in the fluorescence intensity at 518 nm (λ_{ex} = 495 nm). Hence, we could irradiate the 6-carboxyfluorescein encapsulated liposomes at 365 nm (for photocleavage), and monitor their uncorking by measuring the release of the fluorophore at 518 nm (see ESI†).

Fig. 2 shows the plot of the increase in the fluorescence intensity at 518 nm as a function of the irradiation (at 365 nm) time. A control experiment was also performed, in which the liposomes were not irradiated (solid squares).

When we attempted to analyze the cleavage data by a single exponential rate equation, the fit was not good. This was not unexpected since the time course of fluorescence increase involves a finite lag phase. Such a kinetic profile could emerge if the release of the liposome encapsulated fluorophore required some structural adjustments in the liposomal lipid domains. The kinetic data of Fig. 3 could be best fitted by a sequential two step kinetic equation[18] in the following form [eqn. (1)], with k_1 and k_2 values of 0.246 and 0.039 min^{-1}, respectively.

$$\mathrm{L-F} \xrightarrow{k_1} \mathrm{L^*-F} \xrightarrow{k_2} \mathrm{L^*+F}$$
$$\mathrm{F} = [\mathrm{L-F}]\left[1+\left(\frac{1}{k_1-k_2}\right)\left(k_2 e^{-k_1 t}-k_1 e^{-k_2 t}\right)\right] \quad (1)$$

In eqn. (1), L and F represent liposome and 6-carboxyfluorescein (fluorophore), respectively. L^* represents the "intermediary" structure of the liposome, which still harbors the fluorophore in its lumen. The fluorophore is released during the second step. Alternatively, the model mechanism of eqn. (1) can be explained on the basis that the fluorophore exists in the "self-quenched" and "free" states, and the biphasic kinetic profile of Fig. 2 is a result of the transition between such states. Irrespective of the nature of the



Scheme 2 The structures of the products after photolysis of lipid **1** are shown.

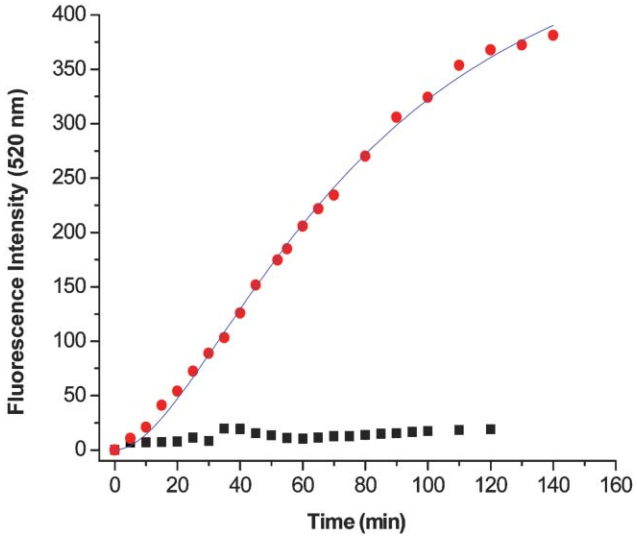


Fig. 2 Kinetics of release of 6-carboxyfluorescein upon irradiation of liposomes incorporating lipid **1** at 365 nm (solid circle). The solid squares represent the control experiment in which the liposomes were not irradiated. The solid smooth line is the best fit of the data for a "two-step" liposomal uncorking according to eqn. (1), for the k_1 and k_2 values of 0.246 and 0.039 min^{-1}, respectively.

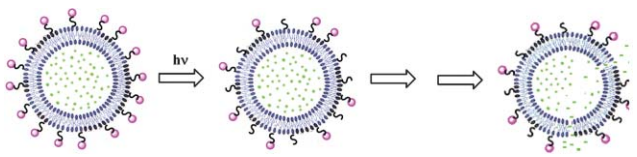


Fig. 3 The photorelease of the liposomal contents is illustrated.

"species" involved in the overall microscopic pathway, it is clear that the rate constant of photocleavage of lipid **1** (0.43 min^{-1}; Fig. 1) is comparable to that of the first step in eqn. (1).

The similarity of the two rate constants suggests that the first step in the release process is the loss of the hydrophilic head group of the lipid **1**. The resultant nitroso benzaldehyde compound (Scheme 2) destabilizes the liposome bilayer. It is possible that lipid reorganization also takes places before the encapsulated dye is released. We are currently in the process of deciphering the kinetic mechanism of the overall process, and we will report these findings subsequently. The contemplated sequence of steps intrinsic to the photocleavage of lipid **1** (incorporated in the liposomes), uncorking of the liposomes, and release of their contents is shown in the cartoon of Fig. 3.

Due to ease of the syntheses of *o*-nitrobenzyl conjugated photocleavable lipids and their abilities to become incorporated in the liposomes, we could demonstrate the feasibility of the photo-induced uncorking of liposomes and release of their contents. The liposomes were found to be stable (in the absence of light) for more than two weeks at 4 °C. The rate of contents release is useful for *in vivo* applications.[10,19] Thus, our overall methodology has the potential to find applications in the area of "drug delivery" in biomedical research.[1]‡

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Notes and references

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