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Lipophilic ruthenium salen complexes: incorporation into liposome bilayers and photoinduced release of nitric oxide†

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A new lipophilic Ru salen complex with cholesterol groups can be efficiently incorporated into liposome bilayers, allowing the photoinduced release of nitric oxide (NO) and the membrane transport of NO to coexisting liposomes.

Nitric oxide (NO) is known to act as a cellular signalling molecule in mammals and to control physiological functions such as vasodilation, smooth muscle relaxation, and platelet aggregation inhibition.[1,2] The development of NO delivery systems is important not only for therapeutic applications but also to provide tools to elucidate signalling mechanisms.[3] Precise spatiotemporal control of NO release is essential because of the short half-life of NO (3–6 s). Because light is the best and least invasive on/off trigger, various photoinduced NO donors have been reported.[4–7] In particular, metal complexes have exhibited high potential as NO donors because of their high stability under physiological conditions and excellent designability. Some metal nitrosyl complexes, such as Cr, Mn, and Ru nitrosyl, have been reported as light-responsive NO precursors.[5–7] However, their low water solubility prevents further application in living systems.

To actualize a biocompatible NO delivery system, assemblies of photoactive metal nitrosyl complexes, using appropriate materials such as polymers, nanoparticles, and vesicles, have been constructed.[8] A spherical vesicle, a liposome consisting of a phospholipid bilayer, is an attractive platform for targeted drug delivery because of its high biocompatibility, long circulation time, and immunomodification.[9] Ford *et al.* reported the liposome encapsulation of a photochemical NO precursor, *trans*-$[Cr(cyclam)(ONO)_2]^+$.[10] However, the encapsulation efficiency of the Cr complex was very low (*ca.* 1%) owing to its limited specific interaction with liposomes. Light-controlled NO release near a membrane surface has not been reported, but would be useful especially for regulation of membrane proteins such as cytochrome c oxidase, which is reversibly inhibited by NO.[11] We therefore designed a new lipophilic Ru nitrosyl complex, [Ru(L)Cl(NO)] (**1**, L = *N*,*N*′-ethylene-bis(4-cholesteryl-hemisuccinate-salicylideneamine)), to fix the Ru nitrosyl complex $[Ru^{II}(salen)Cl(NO)]$[12–14] on the liposome surface through specific hydrophobic interactions between the cholesterol and phospholipid bilayers (Fig. 1).[15,16] The designed lipophilic Ru(salen) complex with cholesterol groups is expected to be efficiently incorporated into liposome bilayers, allowing light-controlled NO release.

Compound **1** was synthesized according to the method described in the ESI,† and identified by elemental analysis and ^{1}H NMR. The UV-vis spectrum of **1** in chloroform (50 μM) showed absorbance at 376 nm, which corresponded to the σ–π* band (Fig. 2a).[12,13] The IR spectrum of **1** exhibited a NO stretch band at around 1833 cm^{-1} (Fig. 2b), which indicated that the coordination geometry of the ruthenium centre in **1** was identical to that of the 'unmodified complex' [Ru(salen)-Cl(NO)] in having a multiple bond between Ru(II) and nitrosonium (NO^+).[14] The NO releasing ability of **1** was determined by measuring the time-dependent spectral changes of a chloroform solution of **1** (50 μM) under Xe irradiation (400–750 nm) at 20 °C (Fig. 2a). The pale brown-coloured solution turned green upon irradiation. New absorption peaks appeared at around 396 nm and 648 nm after irradiation, and their

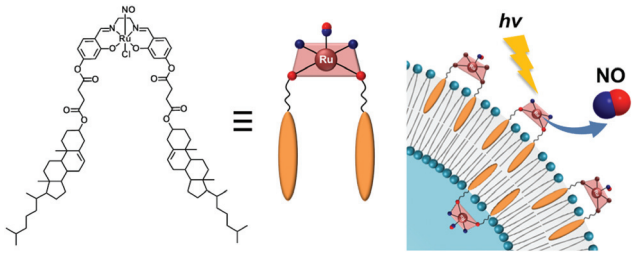

Fig. 1 Structure of **1** and schematic model for incorporation of **1** into liposomes.

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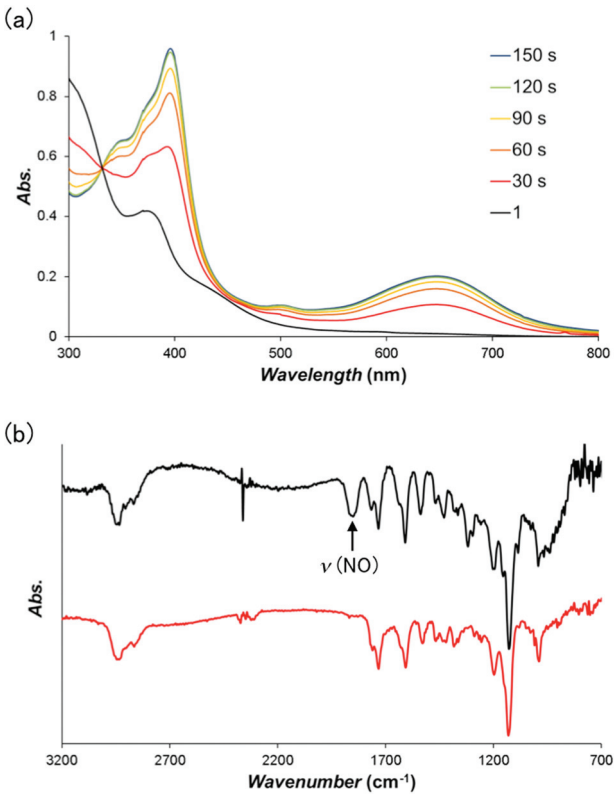

Fig. 2 (a) UV-vis spectra of **1** ([Ru] = 50 μM) in $CHCl_3$ and spectral changes associated with Xe irradiation (400–750 nm) at 20 °C, and (b) IR spectra of **1** (black) and photoproduct (red).

absorbances increased with prolonged irradiation, with an isosbestic point occurring at 331 nm. The broad band around 648 nm was assigned to a ligand-to-metal (Ru^{III}) charge transfer (LMCT) band of the Ru(III) salen complex.[12,13] This spectral change is consistent with the generation of the [Ru^{III}(salen)Cl] species accompanying NO release. After 120 s of Xe irradiation, no significant further spectral changes were observed, and the NO stretching band disappeared in the IR spectrum of the photoproduct (Fig. 2b). These results indicate that modification of the precursor complex [Ru(salen)Cl(NO)] with cholesterol groups did not inhibit the photoinduced NO releasing ability, and **1** in $CHCl_3$ immediately released all captured NO upon Xe irradiation.

A composite of **1** and 1,2-ditetradecanoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (DMPG) liposome (**1**_Lipo) was prepared by incorporation of **1** into a lipid bilayer of the liposome by the Bangham method,[17] in which a lipid film containing DMPG, cholesterol, and **1** in a molar ratio of 20 : 4 : 1 was hydrated with 20 mM Tris/HCl buffer (pH 7.4). The mixture was purified using Sephadex G-25 to afford a pale yellow suspension of **1**_Lipo. The UV-vis spectrum of **1**_Lipo showed a characteristic absorbance at around 376 nm and a broad absorption band from 400 to 800 nm (Fig. 3). These were assigned to the σ–π* band of **1** and light scattering by the liposome suspension, respectively.

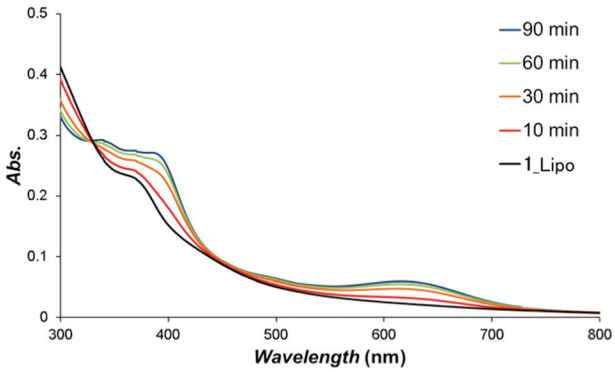

Fig. 3 UV-vis spectra of **1**_Lipo ([Ru] = 11.2 μM) in 20 mM Tris/HCl buffer (pH 7.4) and spectral changes associated with Xe irradiation (400–750 nm) at 20 °C.

The average diameter of **1**_Lipo was determined to be 141 nm by dynamic laser scattering analysis (Fig. S1†). The formation of liposomes was also confirmed by the direct observation of a giant vesicle of **1**_Lipo using confocal laser scanning microscopy (Fig. S2†). The ruthenium and phosphorus concentrations of purified **1**_Lipo were 33 μM and 0.94 mM, respectively. These values mean that the incorporation efficiency of **1** in **1**_Lipo is 66%, and the molar ratio of **1** to DMPG is 1 : 28. The efficiency was significantly improved compared with that of the previously reported liposomal encapsulation of a NO precursor (about 1%).[10] Moreover, the unmodified complex [Ru(salen)Cl(NO)] was not incorporated into liposomes. These results suggest that the Ru cores of **1** were located on the liposome surface with the support of the lipophilic cholesterol groups.

The NO releasing ability of **1**_Lipo under Xe irradiation was evaluated by UV-vis spectroscopy (Fig. 3). As in the case of **1**, the absorbance of new absorption bands at 396 nm and 625 nm (LMCT band) gradually increased during Xe irradiation over a period of 90 min. These spectral changes correspond to the generation of the [Ru^{III}(salen)Cl(H_2O)] species accompanying NO release. The particle size distribution of **1**_Lipo hardly changed after irradiation (Fig. S1†); thus, aggregation and disruption of liposomes did not occur during the reaction. The amount of NO released from **1**_Lipo upon irradiation was confirmed using the fluorescent reagent 4,5-diaminofluorescein (DAF-2). DAF-2 reacts with NO to yield a compound with green fluorescence (DAF-2T) at 515 nm (excitation λ_{max} = 495 nm).[18] The fluorescence spectra of the mixture of **1**_Lipo ([Ru] = 3.3 μM) and DAF-2 (10 μM) showed strong fluorescence at 515 nm after Xe irradiation (Fig. 4). The time profile of the fluorescence at 515 nm showed almost the same curve as that of the absorbance at 396 nm in the UV-vis spectra (Fig. S3†). This result indicates that the NO released from **1**_Lipo immediately diffused in the solution, and reacted with DAF-2. The concentration of released NO after 60 min irradiation was 2.3 μM, indicating that about 70% of **1** in **1**_Lipo was converted to the photoproduct. The micromolar-

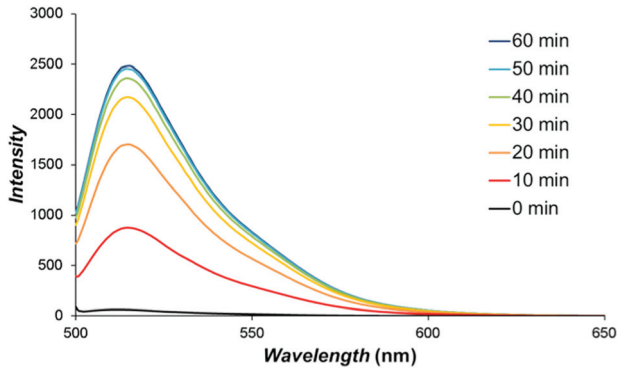

Fig. 4 Fluorescence spectra of the mixture of **1**_Lipo ([Ru] = 3.3 μM) and DAF-2 (10 μM) in 20 mM Tris/HCl buffer (pH 7.4) and spectral changes associated with Xe irradiation (400–750 nm) at 20 °C (λ_{ex} = 495 nm).

order concentration could be sufficient to control biological activities such as anticancer activity.[5]

DAF-2-encapsulated liposome (DAF-2@Lipo) was then prepared to examine transport of the NO from **1**_Lipo to other liposomes (Fig. 5). The fluorescence spectra of a mixture of **1**_Lipo and DAF-2@Lipo showed a gradual increase in fluorescence intensity resulting from the reaction between NO and DAF-2 under Xe irradiation (Fig. 5b). Confocal laser scanning microscopy images of the mixture of **1**_Lipo and DAF-2@Lipo before and after irradiation are shown in Fig. 5c. After irradiation, green fluorescence was observed only in the interior aqueous phase of DAF-2@Lipo, indicating the successful membrane transport of NO from **1**_Lipo to the coexisting DAF-2@Lipo.

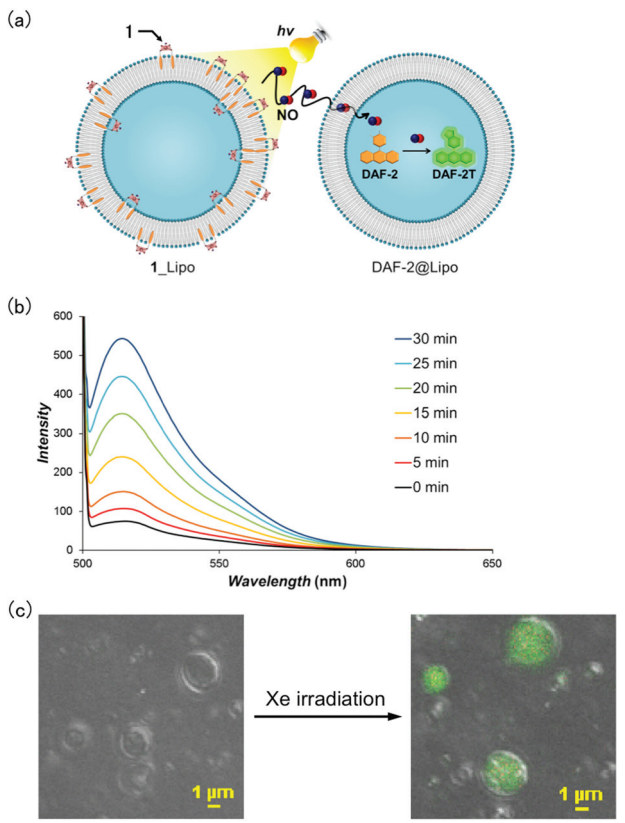

Fig. 5 (a) Schematic representation of NO transport from **1**_Lipo to DAF-2@Lipo, (b) fluorescence spectra of the mixture of **1**_Lipo and DAF-2@Lipo in 20 mM Tris/HCl buffer (pH 7.4) and spectral changes associated with Xe irradiation (400–750 nm) at 20 °C (λ_{ex} = 495 nm), and (c) confocal laser scanning microscopy images of the mixture of **1**_Lipo and DAF-2@Lipo before and after irradiation.

In conclusion, we have demonstrated the photoinduced release of NO from liposomes incorporating the lipophilic Ru salen complex **1**. The efficient incorporation of **1** into liposome bilayers was achieved as a result of the water insolubility of **1** and the high affinity of the cholesterol groups for phospholipid bilayers. We consider our strategy to be a rational approach for applying various water-insoluble photoinduced NO donors in aqueous media. The composite of **1** and liposome gradually released NO during Xe irradiation over a period of 90 min, with relatively high efficiency. Furthermore, we succeeded in achieving the membrane transport of NO to coexisting liposomes. We have reported that the reactivity of metal complexes on a liposome surface is influenced by the surrounding environment of the reaction centre, including the position of metal cores and the head groups of phospholipids.[16] Thus, further adjustment of the surroundings of lipophilic Ru nitrosyl complexes could allow control of the rate and concentration of released NO, leading to elucidation of the mechanisms of biological effects such as vasorelaxant and anticancer effects.[5]

Acknowledgements

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