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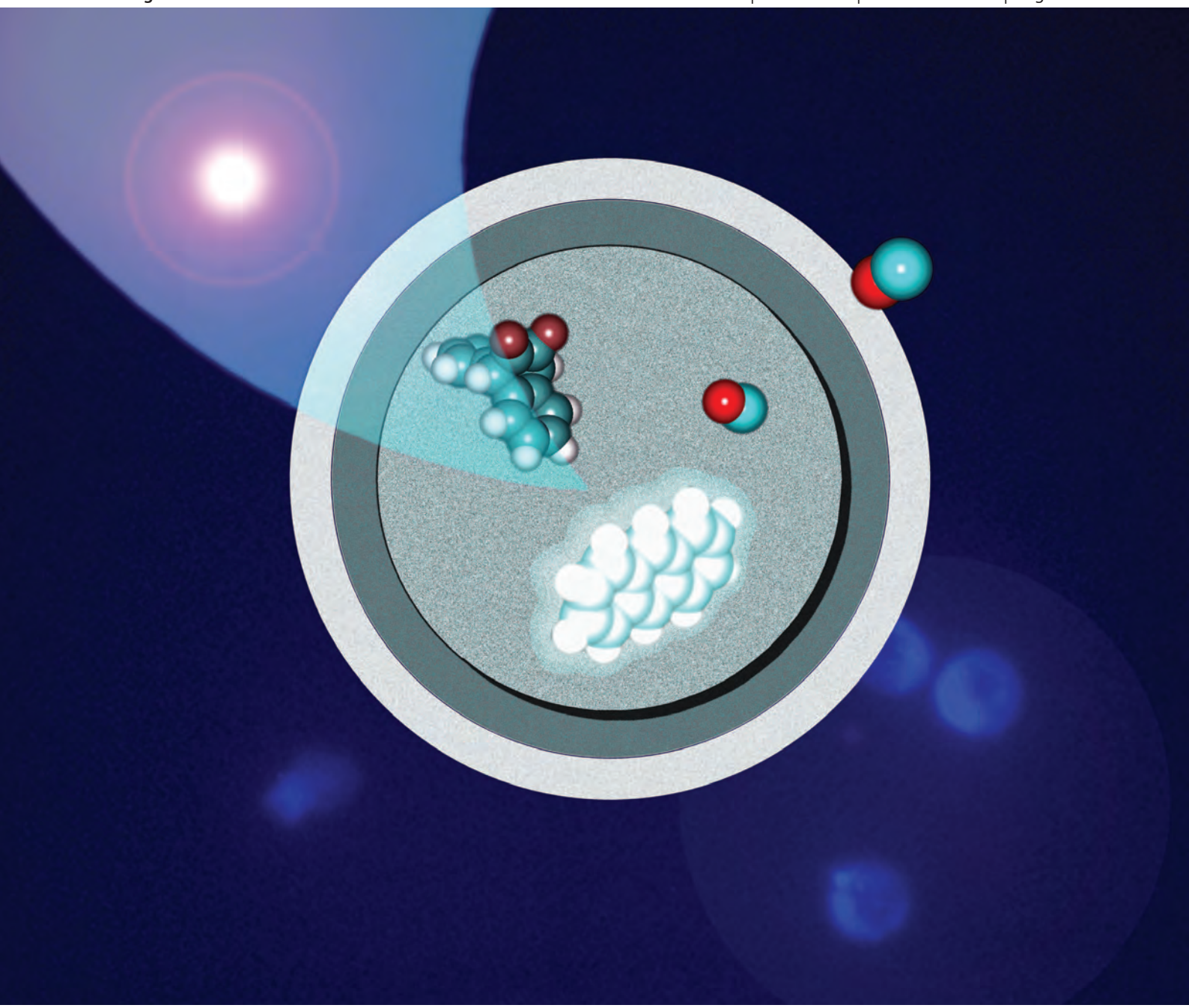

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Visible-light activatable organic CO-releasing molecules (PhotoCORMs) that simultaneously generate fluorophores†

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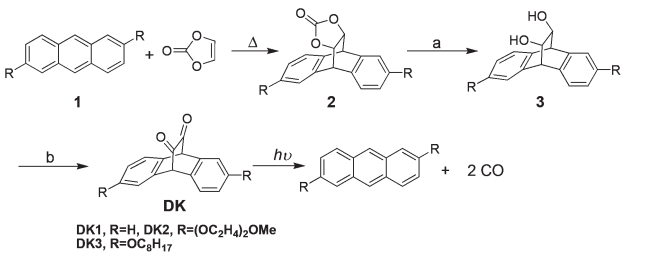
Novel organic photoCORMs based on micelle-encapsulated unsaturated cyclic α-diketones were designed and synthesized. These photoCORMs can be activated by visible light, have potentially low toxicity, allow the delivery of carbon monoxide to be monitored by fluorescence imaging techniques, and thus are useful tools for the study of the biological function of CO.

The use of gaseous molecules in biological and therapeutic fields has attracted great attention in recent years. For instance, it has been shown that nitric oxide (NO) is involved in the cellular processes of the cardiovascular and nervous systems, and can be used to control blood pressure.[1] Hydrogen sulphide (H_2S), produced from L-cysteine of mammalian cells, has shown anti-apoptotic and anti-inflammatory effects, and novel H_2S releasing molecules have been developed.[2] Carbon monoxide (CO) is another gaseous molecule that plays a profound and important role in regulating cell functions.[3] Compared with NO and H_2S, CO has relatively low chemical reactivity and thus could be easier to use for medical purposes than NO and H_2S. The beneficial properties of CO have been confirmed in the models *in vitro* and *in vivo*, showing that CO reduces the inflammation associated with allergen-induced asthma in mice, protects against orthotopic lung transplantation and lung injury caused by oxidants, and reverses established pulmonary hypertension.[4–6]

Due to the inherent toxic nature of CO, it is critical to control the dose and site of CO release. CO-releasing molecules or materials (CORMs) capable of carrying and releasing CO in cellular systems are promising alternatives in the attempt to overcome the limitations of CO gas.[7] Most of the CORMs reported previously release CO by hydrolysis under physiological conditions. The *in vivo* application is often limited by the short half-life of these CORMs.[8] Some metal carbonyl complexes such as $Mn_2(CO)_{10}$ and $[Mn(CO)_3(tpm)]PF_6$ release CO upon photo-irradiation.[9] This is a desirable property since it promises precise spatial and temporal control of CO release. Reviews on photo-activatable CORMs (photoCORM) have been published recently.[10] However, these photoCORMs need ultra-violet (UV) light to release CO, which is apparently undesirable for biological applications. Herein, we report that unsaturated cyclic α-diketones encapsulated in micelles are effective CORMs that can be activated by visible light in contrast to UV light. Unlike most of the previously developed CORMs (which are metal complexes and organoboron compounds[11]), the materials reported here are organic compounds, which promise easy modification, likely cellular uptake, and relatively low toxicity.

The unsaturated cyclic α-diketones (DKs) were prepared as shown in Scheme 1. Anthracene derivatives reacted with vinylene carbonate to form cyclic Diels–Alder adducts. The Diels–Alder adducts were hydrolyzed in NaOH solution to form the dihydroxy compounds, which were oxidized to DKs *via* Swern oxidation reactions. Photochemical reaction of DK1 (9,10-dihydro-9,10-ethanoanthracene-11,12-dione) has previously been studied.[12] The short PEG chains on DK2 [2,6-bis(2-(2-



Scheme 1 Syntheses and photoreactions of DKs: (a) NaOH–dioxane, reflux, 2 h; (b) DMSO, TFAA, Et_3N, DCM, −78 °C.

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†Electronic supplementary information (ESI) available: Material, experimental details, micelle preparation, CO detection using two-compartment myoglobin test, CO detection using the Rh complex, cell biology tests. See DOI: 10.1039/c3ob41385c

methoxyethoxy)ethoxy)-9,10-dihydro-9,10-ethanoanthracene-11,12-dione] increase the hydrophilicity of DK2 and enable it to be dissolved in a DMSO–water mixture. In contrast, the $-OC_8H_{17}$ side chains on DK3 [2,6-bis(octyloxy)-9,10-dihydro-9,10-ethanoanthracene-11,12-dione] decrease the hydrophilicity of the diketone.

This type of diketone is known to undergo a photoreaction that releases CO (Scheme 1), and the mechanism has been previously studied.[13] Upon irradiation at a wavelength in the absorption band of its n–π* transition (400–550 nm), the diketones release two CO molecules and generate anthracene derivatives. It is worth mentioning that unlike many polycyclic aromatic hydrocarbons, anthracene is not acutely toxic, carcinogenic, or mutagenic.[14] Although anthracene derivatives could be more or less toxic than anthracene, it is reasonable to expect that the remaining chemicals after CO release could have low toxicity, which is one of the advantages of these CORMs. Indeed, as described below, a cell viability test showed that the synthesized derivatives have low cell toxicity.

The photochemical reactions of DKs are examined in DMSO, acetonitrile and dichloromethane (DCM) solutions. UV-vis spectra showed that the expected photoreactions occurred for all the three compounds. The absorption spectrum of DK3 in DMSO is shown in Fig. 1. The absorption of the n–π* transition is between 400 nm and 550 nm with a maximum (λ_{max}) at 465 nm. After irradiation with a 470 nm LED array (Elixa Ltd) for 10 min, the absorption of DK3 disappeared completely and the UV-vis spectrum matched that of 2,6-bis(octyloxy)anthracene which is the product after CO is released from DK3 (Scheme 1), indicating that the photoreaction occurred quantitatively in the organic solvents. NMR study of the photoreaction in organic solvents also confirmed the production of the expected anthracene derivatives.

To test the CO releasing capability of DKs in aqueous media, DK2, which has two hydrophilic PEG chains, was dissolved in a DMSO (1%)–water mixture. (One per cent DMSO was added to increase the solubility of the compound in water.) The absorption peak at 465 nm in DMSO disappeared in the UV-vis spectrum of the solution (Fig. 2), and photoirradiation at 470 nm, 419 nm, 365 nm and 254 nm did not generate the corresponding anthracene derivative. Addition of DMSO to the solution regenerated the absorption peak at

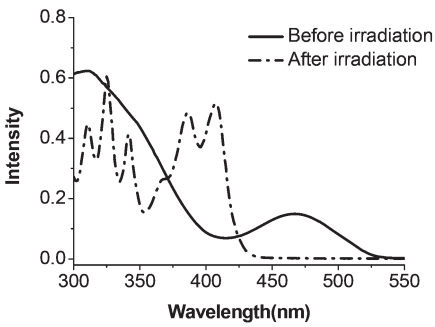

Fig. 1 Absorption of DK3 in DMSO solution before and after irradiation at 470 nm.

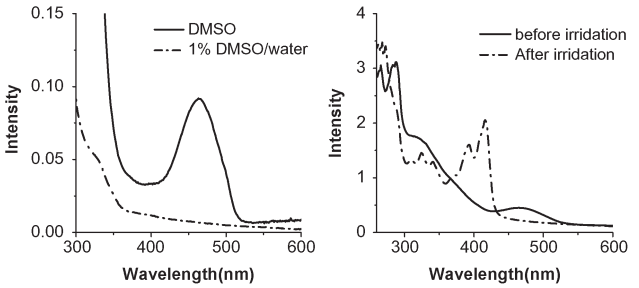

Fig. 2 UV-vis spectrum of DK2 in DMSO and a 1% DMSO–water mixture (left) and UV-vis spectrum of aqueous solutions of Pluronic encapsulated DK3 before and after irradiation (right).

465 nm, which indicates that the deactivation of the diketone is most likely due to reversible hydration of the carbonyl groups. All ketones form equilibrium with the corresponding hydrates in water, in which the ketone form is often predominant. However, in the α-diketones, one of the carbonyl groups is activated by the other one, which shifts the equilibrium to the hydrates. The hydrates do not possess the n–π* transition (400–550 nm) of the diketones, and thus show no absorption peaks in the visible range.

To solve this problem, DKs were encapsulated in Pluronic F127 micelles by following a literature method.[15] Pluronics are biocompatible block copolymers of polyethylene oxide and polypropylene oxide, which have been widely used as carriers for drug delivery.[16] The inner environment of Pluronic micelles is hydrophobic and thus can protect the DKs from hydration. In addition, encapsulating DKs in Pluronic micelles allows all the three DKs, including the hydrophobic DK3, to be dissolved in water without the addition of DMSO. As shown in Fig. 2, Pluronic encapsulated DKs showed a 400–550 nm absorption band in their UV-vis spectra. After irradiating the aqueous solutions of encapsulated DKs (407 μM) for 10 min, the 400–550 absorption band disappeared, and absorption peaks for the corresponding anthracene derivatives appeared.

To determine the yield of the photoreactions, DMSO was added and the UV-vis spectra of the resulting solutions were compared to those of the DMSO solutions of the corresponding anthracene derivatives at the same concentration. The photoreaction yields for the micelles of DK1, DK2 and DK3 were 78%, 71% and 90%, respectively. Unlike the reactions in organic solvents, these reactions did not give quantitative yields, which indicates that encapsulation with Pluronic does not completely stop hydration of the diketones. DK2, which is the most hydrophilic one, showed the lowest yield, and DK3, which is the most hydrophobic one, showed the highest yield.

CO released from CORMs is often measured using a solution of myoglobin Fe(II) [MbFe(II)], freshly reduced with excess sodium dithionite under nitrogen.[7a] However, we found that the diketones react with sodium dithionite, so an *in situ* measurement of CO with MbFe(II) is not possible. To solve this problem, we developed a two-compartment myoglobin test, which is described in detail in the ESI.† In this test, the DKs

were irradiated in an air-tight syringe that was connected to the MbFe(II) solution. After irradiation, an excess amount of the myoglobin solution was drawn in the syringe and reacted with CO. The concentration of MbCO was calculated based on UV-vis absorption of the solution.[7g] Using this method, we calculated that 84% of CO from DK3 bound to myoglobin, which is close to the yield (90%) obtained by measuring the UV absorption of the anthracene derivative.

Release of CO was also qualitatively confirmed by using the CO sensitive Rh complex recently reported by Esteban and co-workers.[17] *cis*-$[Rh_2(C_6H_4PPh_2)_2(O_2CCH_3)_2](HO_2CCH_3)_2$ dispersed on silica gel is highly sensitive to CO. Therefore, we sealed some silica gel powders absorbed with the Rh complex in a side arm of a round bottom flask filled with an aqueous solution of DK3 micelles. After irradiating the micelle solution, the color of the Rh complex changed from violet to orange, which is consistent with the color change reported in the literature (Fig. S2†). A reflectance UV-vis spectrum of the powder showed that a mono-CO complex formed after the reaction (ESI†).

Another advantage of this type of CORM is that the anthracene derivatives generated simultaneously with CO are fluorophores, which allows fluorescence imaging of the studied cells. For example, anthracene has a high fluorescence quantum yield of 0.36.[18] To demonstrate this advantage, micelles of DK3 were incubated with acute myeloid leukemia (AML) cells KG-1. The KG1 cells were derived from an AML patient and are a suspension of cells. The following day, cells were irradiated with a single wavelength of light (λ = 470 nm) of six 30 second pulses. Photo-activation of the CORM was assessed by fluorescence microscopy (Fig. 3). The fluorescence image showed bright blue fluorescence in the cells, originating from the emission of the corresponding anthracene derivative. These results confirmed that DK3 were taken up by the cells and photo-induced CO release occurred in the cells.

Cell proliferation and viability were monitored over a 3 day period by directly measuring the cell growth and viability by flow cytometry (Fig. 3 and Fig. S6†). No differences were observed in the viability of cells exposed to micelles of DK3 and light activation as compared to control cells. Neither the photoCORM nor the anthracene based fluorophore (up to 40 μM) had an effect on cell viability as assessed by measuring the number of apoptotic and necrotic cells by flow-cytometry. Furthermore, no photodamage was observed under the experimental conditions. Thus, this system is well suited for possible targeted delivery of gasotransmitter carbon monoxide to biological systems to study its function in signalling as well as for potential therapeutic applications. The detailed experimental procedure and data are given in the ESI.†

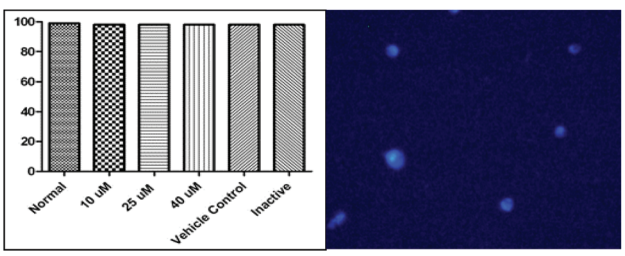

Fig. 3 Cell viability for DK micelles (normal: KG1 cells only, vehicle control: KG1 cells with Pluronic, inactive: KG1 cells with the anthracene derivative), and fluorescence image of the cells incubated with DK3 micelles and irradiated with 470 nm light (right). (More fluorescence images are provided in the ESI.†)

Conclusions

In conclusion, photoCORMs based on micelle encapsulated diketones were synthesized and their CO releasing capability was studied. Although unsaturated cyclic α-diketones are known to release CO upon irradiation, they are not photoactive under physiological conditions. Micelle-encapsulation allows the diketones to be activated by visible light under physiological conditions. In addition, these organic CORMs allow the delivery of CO to be monitored by fluorescence imaging techniques, have potentially low toxicity, and thus can be useful tools for the study of the biological function of CO.

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