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Improved photodynamic activities of liposome-incorporated [60]fullerene derivatives bearing a polar group†

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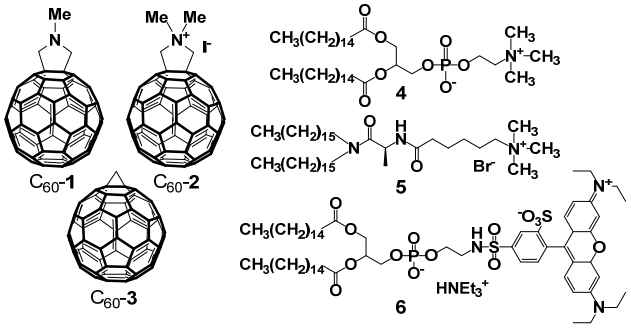
[60]Fullerene (C_{60}) derivatives were incorporated into liposomes using a fullerene exchange method involving the transfer of the fullerene from the cavity of two *γ*-cyclodextrin molecules to a liposome. A lipid-membrane-incorporated C_{60} derivative bearing a polar group showed much higher photodynamic activity than the analogous system incorporating pristine C_{60}.

Photodynamic therapy (PDT) is a promising approach for the treatment of malignant tumours and macular degeneration.[1] The photosensitisers (PSs) used in PDT are activated by light irradiation and transfer their energy to nearby oxygen molecules ($^3O_2$) to form singlet oxygen ($^1O_2$), which results in irreversible damage to the tumour cells.[1,2] Fullerenes[3] and their derivatives[4,5] are efficient visible light triplet-sensitisers and exhibit high photoproduction ability for $^1O_2$.[6] In particular, the *N*,*N*-dimethylpyrrolidinium derivatives of [60]fullerenes (C_{60}) complexed with *γ*-cyclodextrins (*γ*-CDxs) have higher photodynamic activities than pristine C_{60}, as well as several other C_{60} derivatives and photofrin, which is commonly used in clinical practice for PDT.[7] The incorporation of the C_{60} derivatives into the lipid membranes ($LMIC_{60}$ derivatives) would lead to the formation of $LMIC_{60}$ derivatives with high photodynamic activities. However, lipid-membrane-incorporated guest molecules can be difficult to prepare using guest molecules with a high dipole moment, because some of the guest molecules precipitate from solution or leak into the bulk water.[8] In an attempt to solve these problems, it was envisaged that lipid membrane-incorporated C_{60} derivatives ($LMIC_{60}$-derivatives) could be prepared based on the exchange reaction of a functionalised C_{60} from a *γ*-CDx•C_{60} derivative complex[9] to a liposome, which we defined as the 'exchange method' (Scheme S1).[3a,c] $LMIC_{60}$-derivatives would be expected to have high photodynamic activities because of the high photodynamic activities of the *γ*-CDx•C_{60} derivative complexes. Furthermore, the results of molecular dynamics simulations of fullerenes inside the lipid bilayer have shown that the C_{60} molecules exist between the two membranes of the liposomes.[10] We have confirmed these results experimentally.[11] These reports suggest that $^3O_2$ molecules have to migrate into the membranes to come into contact with photoexcited fullerenes before being converted to $^1O_2$ through an energy transfer process. The introduction of a C_{60} derivative in close proximity to the surface of the liposome should therefore lead to an increase in the photodynamic activity by enhancing the likelihood of there being contact between $^3O_2$ and the photoactivated C_{60} derivative. In this paper, we describe the preparation of three lipid membrane-incorporated C_{60} systems, including pyrrolidine, *N*,*N*-dimethylpyrrolidinium iodide and methylene derivatives of C_{60} ($LMIC_{60}$-**1**–C_{60}-**3**).[12] All three of these systems were prepared using the exchange method and



their biological activities were evaluated under visible light irradiation. The results revealed that $LMIC_{60}$-**2** showed a dramatic improvement in photodynamic activity compared with pristine C_{60}.

$LMIC_{60}$-**1**–C_{60}-**3** were prepared using a fullerene exchange reaction between the liposomes and the *γ*-CDx•C_{60}-**1**, C_{60}-**2** and C_{60}-**3** complexes[9] by heating at 80°C for 1 h, as described in our

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previous report (Scheme S1a).[3c] The size distributions of the liposomes were studied using dynamic light scattering (DLS). Table S1 shows that the hydrodynamic diameters (D_{hy}) changed from 110–145 nm before the exchange reactions to 105, 138 and 112 nm for LMIC$_{60}$-**1**, C$_{60}$-**2** and C$_{60}$-**3**, respectively, indicating that the incorporation of the fullerene derivatives had very little impact on the size of the liposomes. The ^{1}H NMR peaks assigned to the *γ*-CDx•C$_{60}$-**1**, C$_{60}$-**2**, and C$_{60}$-**3** complexes disappeared completely after the C$_{60}$ derivative-exchange reactions (Figs. 1, S1 and S2). These results indicated that all of the C$_{60}$-**1**, C$_{60}$-**2** and C$_{60}$-**3** molecules had been released from the *γ*-CDx cavities in the presence of the liposomes. Furthermore, it is well known that all of the peaks belonging to the guest molecules and the lipids disappear completely in these systems as a consequence of peak broadening following the formation of the liposomes. The disappearance of the peaks belonging to the C$_{60}$-**1** and C$_{60}$-**2** molecules in the current study therefore suggests that all of these molecules were completely incorporated into the liposomes (Fig. 1, blue circles). In contrast, the rapid mixing of an aqueous solution of the *γ*-CDx•C$_{60}$-**2** complex with a solution of liposomes solution resulted in a new peak (5.4 ppm). This peak was attributed to a self-aggregate of C$_{60}$-**2** coated with *γ*-CDx, which was similar to that of the self-aggregated *γ*-CDx•C$_{60}$ complex obtained by heating (5.41 ppm) (Fig. S2d).[3a] This result indicated that part of the C$_{60}$-**2** molecule self-aggregated outside the liposome. We therefore investigated the dropwise addition of an aqueous solution of *γ*-CDx•C$_{60}$-**2** complex to an aqueous solution of liposomes (Scheme S1b). ^{1}H NMR showed that the peak at 5.4 ppm associated with the self-aggregation of C$_{60}$-**2** did not appear (Fig. S2e), indicating that almost all of the C$_{60}$-**2** molecules had been successfully incorporated into the lipid membranes, resulting in the formation of LMIC$_{60}$-**2**.

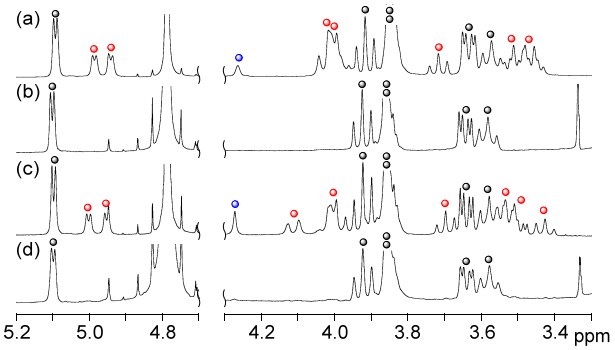

Fig. 1 Partial ^{1}H NMR spectra of the *γ*-CDx•C$_{60}$-**1** complex (a) before and (b) after the addition of liposome-**4**-**5** and *γ*-CDx•C$_{60}$-**2** complex (c) before and (d) after the addition of liposome-**4**-**5** (●: free *γ*-CDx, ●: *γ*-CDx in the *γ*-CDx•C$_{60}$ derivative complex, ●: C$_{60}$ derivative in the *γ*-CDx•C$_{60}$ derivative complex, [C$_{60}$-**1** or C$_{60}$-**2**]/[**4** + **5**] = 5 mol%, [**4** + **5**] = 1.0 mM).

The UV-vis absorption spectra of LMIC$_{60}$-**1**–C$_{60}$-**3** exhibited broad absorption bands in the range of 200–600 nm (Figs. 2a and S3), which were absent from their cyclodextrin complexes, indicating that C$_{60}$-**1**, C$_{60}$-**2** and C$_{60}$-**3** formed self-aggregates in the lipid membranes. These results were similar to that of the LMIC$_{60}$ material prepared by the exchange method at 80 °C. However, because the spectrum of LMIC$_{60}$-**2** contained sharper peaks than those of LMIC$_{60}$-**1** and LMIC$_{60}$-**3**, this result suggested that C$_{60}$-**2** self-aggregated to a much lesser extent than C$_{60}$-**1** or C$_{60}$-**3**. This difference was mainly attributed to electrostatic repulsion between the cationic C$_{60}$-**2** molecules.

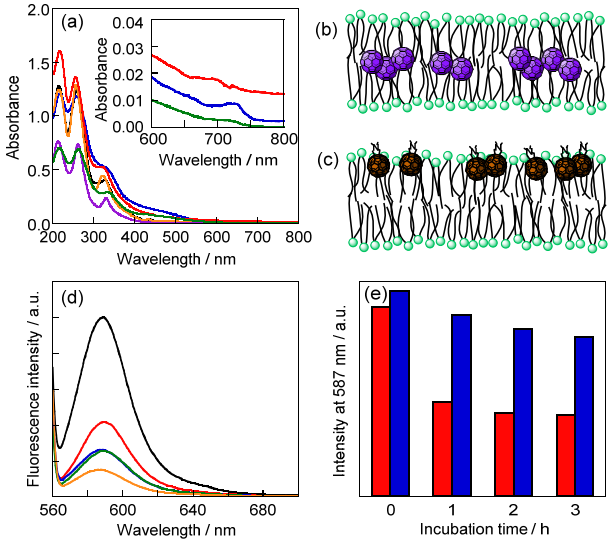

Fig. 2 (a) UV-vis absorption spectra of the *γ*-CDx•C$_{60}$-**1** (black line), *γ*-CDx•C$_{60}$-**2** (orange line) and *γ*-CDx•C$_{60}$-**3** (purple line) complexes and LMIC$_{60}$-**1** (blue line), LMIC$_{60}$-**2** (red line) and LMIC$_{60}$-**3** (green line) after the exchange reaction. The inset shows the 600–800 nm regions of LMIC$_{60}$-**1** (blue line), LMIC$_{60}$-**2** (red line) and LMIC$_{60}$-**3** (green line) ([C$_{60}$-**1**, C$_{60}$-**2** or C$_{60}$-**3**]/[**4** + **5**] = 5 mol%, [**4** + **5**] = 2.0 mM). All of these spectra were recorded at 25 °C with a 1 mm cell. Schematic illustrations of (b) C$_{60}$ and (c) C$_{60}$-**2** in the lipid membranes. (d) Fluorescence spectra (λ_{ex} 550 nm) of liposome-**4**-**5**-**6** (black line) and RhB-LMIC$_{60}$ (red line), RhB-LMIC$_{60}$-**1** (blue line), RhB-LMIC$_{60}$-**2** (orange line) and RhB-LMIC$_{60}$-**3** (green line) consisting of liposome-**4**-**5**-**6**. ([**6**]/[**4** + **5**] = 0.25 mol%, [**5**]/[**4** + **5**] = 10 mol%, [C$_{60}$, C$_{60}$-**1**, C$_{60}$-**2** or C$_{60}$-**3**]/[**4** + **5**] = 5 mol%). (e) Incubation time-dependent fluorescence intensities at 587 nm (λ_{ex} 550 nm) of liposome-**4**-**6** and an RhB-LMIC$_{60}$-**2** mixture consisting of liposome **4** (red bars) and liposome-**4**-**5**-**6** and RhB-LMIC$_{60}$-**2** (blue bars) mixture consisting of liposome-**4**-**5**, respectively. [**6**]/[**4** or **4** + **5**] = 0.25 mol%, [C$_{60}$-**2**]/[**4** or **4** + **5**] = 5 mol%, [**5**]/[**4** + **5**] = 10 mol%.

We have shown experimentally that C$_{60}$ exists between the two membranes of liposomes (Fig. 2b).[11] Furthermore, these finding were consistent with the results predicted by molecular dynamics simulations for the fullerenes inside the lipid bilayer (Fig. 2b).[10] In contrast, molecular dynamics simulations for C$_{60}$ derivative bearing hydrophilic moieties on a half sphere revealed that this material most likely exists on the hydrophilic surface of the liposome (Fig. 2c).[10b] It was therefore envisaged that a large number of $^1O_2$ would be produced by an effective energy transfer process from the photo-excited C$_{60}$-**2** molecules to the dissolved oxygen molecules because of the increasing number of collisions. To directly confirm the location of these fullerenes, we measured fluorescence quenching effects of rhodamine B-dipalmitoyl phosphatidylethanolamine (**6**) on the fullerenes in the liposome of **4** containing 0.25 mol% **6** (RhB-LMIC$_{60}$, RhB-LMIC$_{60}$-**1**, RhB-LMIC$_{60}$-**2** and RhB-LMIC$_{60}$-**3**) (Fig. 2d). RhB-LMIC$_{60}$, RhB-LMIC$_{60}$-**1**, RhB-LMIC$_{60}$-**2** and RhB-LMIC$_{60}$-**3** led to fluorescence quenching activities of 58, 74, 85 and 75%, respectively, indicating that C$_{60}$-**2** with a higher hydrophilicity was positioned much closer to the rhodamine moiety than C$_{60}$,

C_{60}-**1** or C_{60}-**3** (i.e., C_{60}-**2** exists in close proximity to the head groups of the lipids).

We wanted to confirm whether C_{60}-**2** was released from the lipid membranes because of the hydrophilicity of C_{60}-**2** (Scheme S2a). C_{60}-**2** was found to be insoluble in water in the absence of a polar organic solvent such as DMSO.[13] As shown in Fig. 2d, the fluorescence of RhB-LMIC$_{60}$-**2** was considerably quenched. Therefore, if C_{60}-**2** was released from the lipid membranes of LMIC$_{60}$-**2** to move in amongst liposome-**4**-**6**, the fluorescence characteristics of liposome-**4**-**6** would be quenched after mixing with the LMIC$_{60}$-**2** solution because C_{60}-**2** molecules released during this process would move into the lipid membranes of liposome-**4**-**6**. The fluorescence of **6** decreased slowly after the mixing of the LMIC$_{60}$-**2** with solutions of liposome-**4**-**6** solutions (Fig. 2e, red bars). However, this decrease implied that (i) the C_{60}-**2** molecules were migrating into the lipid membrane of liposome-**4**-**6** (Scheme S2a); and (ii) the membrane fusion and fission processes were being repeated between LMIC$_{60}$-**2** and liposome-**4**-**6** (Scheme S2b). In an attempt to supress the fusion and fission processes using electrostatic repulsion, we added the cationic lipid **5** ([**5**]/[**4** + **5**] = 10 mol%) to the lipid membrane of LMIC$_{60}$-**2** and liposome-**4**-**6**. This process inhibited the quenching (Fig. 2e, blue bars), which suggested that most of the C_{60}-**2** molecules had not been transferred from LMIC$_{60}$-**2** to liposome-**4**-**5**-**6** (i.e., very little C_{60}-**2** had been released from the lipid membranes) (Scheme S2c).

The photodynamic activity of LMIC$_{60}$-**1**–C_{60}-**3** was evaluated in HeLa cells using a cationic lipid **5** ([**5**]/[**4** + **5**] = 10 mol%).[14] A cationic lipid was selected for this experiment because LMIC$_{60}$ bearing a cationic liposome surface efficiently induced cell death under photoirradiation, whereas LMIC$_{60}$ systems bearing a neutral or anionic liposome surface had no discernible effect.[3] LMIC$_{60}$ and photofrin were used as control samples. After incubation with each LMIfullerene ([fullerene]/[lipids] = 5 mol%), the cells were exposed to light at a wavelength in the range of 610–740 nm. No cytotoxicity was observed when all of the LMIfullerenes were added to the cells without light exposure (Fig. 3a). Furthermore, no photodynamic activity was observed when LMIC$_{60}$, LMIC$_{60}$-**1** or LMIC$_{60}$-**3** was used in combination with light irradiation for wavelengths in the range of 610–740 nm (Fig. 3b, black, blue and green lines). In contrast, the photodynamic activity of LMIC$_{60}$-**2** was much higher than those of LMIC$_{60}$, LMIC$_{60}$-**1** and LMIC$_{60}$-**3** (Fig. 3b, red line). We determined that the medium inhibitory concentration (IC_{50} value) was 1.1 μM for LMIC$_{60}$-**2** ([C_{60}-**2**]/[lipids] = 5 mol%, [lipids] = 0.25–5.00 μM), as shown in Fig. 3b (red line). The IC_{50} value of LMIC$_{60}$-**2** was lower than that of photofrin, which gave an IC_{50} value of 2.1 μM under the same conditions (Fig. S4b) when the number of moles was converted to the number of porphyrin units because photofrin consists of porphyrin oligomers containing two to eight units (Fig. S4a). These results therefore revealed that the photodynamic activity of LMIC$_{60}$-**2** was approximately two times higher than that of photofrin.

We wanted to determine why the photodynamic activity of LMIC$_{60}$-**2** was higher than that of LMIC$_{60}$. In this regard, there are two possible explanations, including (i) LMIC$_{60}$-**2** could improve intracellular uptake by the formation of electrostatic interactions with the anionic surface of the cells, with cationic C_{60}-**2** leading to an increase in the cationic density of the

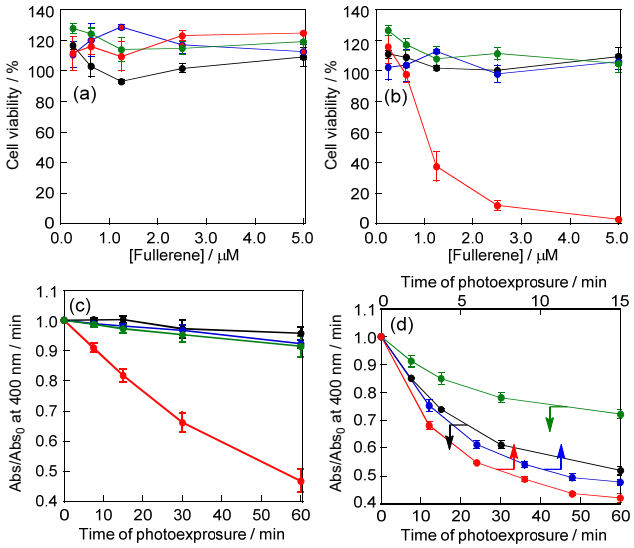

Fig. 3 Concentration-dependent cytotoxicities of LMIC$_{60}$ (black line), C_{60}-**1** (blue line), C_{60}-**2** (red line) and C_{60}-**3** (green line) (a) without and (b) with light irradiation (610–740 nm, 30 min). Cell viability was assayed by the WST-8 method. (c) Detection of $^1O_2$ generation for LMIC$_{60}$ (black circles), LMIC$_{60}$-**1** (blue circles), LMIC$_{60}$-**2** (red circles) and LMIC$_{60}$-**3** (green circles) with light irradiation at wavelengths greater than 620 nm. (d) Detection of $^1O_2$ generation for γ-CDx•C_{60} complex (black circles), γ-CDx•C_{60}-**2** complex (blue circles), LMIC$_{60}$ (green circles) and LMIC$_{60}$-**2** (red circles) with light irradiation at wavelengths greater than 350 nm by the ABDA bleaching method. A DMSO solution of ABDA was injected into an aqueous solution of the liposomes or γ-CDx complexes. The bleaching of ABDA was monitored as a reduction in the absorbance of ABDA at 400 nm. All data represent the mean values of three independent experiments. Error bars represent the standard deviations.

liposomal surfaces;[3b,14] and (ii) the formation of cytotoxic reactive oxygen species (ROS) could increase as a consequence of the effective energy or electron transfer between C_{60}-**2** and the dissolved oxygen molecules. To investigate the validity of explanation (i), we determined the surface potentials of liposomes **3**-**4** in the absence and presence of C_{60}, C_{60}-**1**, C_{60}-**2** and C_{60}-**3** using zeta potential measurements. The results of these experiments are summarised in Table S2. The zeta-potential of liposomes **4**-**5** changed from +41 to +24–35 mV, respectively, following the addition of the fullerenes ([fullerenes]/[lipids] = 5 mol%), indicating that the surface densities of LMIC$_{60}$-**2** were largely unaffected by the addition of cationic C_{60}-**2** compared with the other fullerenes. To test the validity of explanation (ii), we measured the level of $^1O_2$ generation according to a chemical method using 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA)[15] as a detector. This experiment was conducted to clarify the reason for the differences observed in the biological activities of LMIC$_{60}$, LMIC$_{60}$-**1**, LMIC$_{60}$-**2** and LMIC$_{60}$-**3**. The absorption of ABDA at 400 nm (absorption maximum for ABDA) was monitored as a function of time after the irradiation of the samples with light at a lipid concentration of 150 μM (Fig. 3c). The results revealed severe time-dependent bleaching of ABDA for LMIC$_{60}$-**2** compared with LMIC$_{60}$, LMIC$_{60}$-**1** and LMIC$_{60}$-**3**, indicating that much higher levels of $^1O_2$ were generated in LMIC$_{60}$-**2** compared with LMIC$_{60}$, LMIC$_{60}$-**1** and LMIC$_{60}$-**3**. We previously reported

that the photodynamic activity of the *γ*-CDx•C_{60}-**2** complex is much higher than that of *γ*-CDx•C_{60}-**1** complex for wavelengths in the range of 610–740 nm because of the quenching effect of the lone pair of electrons on the amino group.[7] For comparison, we measured the generation of $^1O_2$ in $LMIC_{60}$-**3**, which does not have any amino groups. However, the amount of $^1O_2$ generated by $LMIC_{60}$-**3** was much lower than that of $LMIC_{60}$-**2**, but similar to those of the $LMIC_{60}$ and $LMIC_{60}$-**1** complexes (Fig. 3c). Furthermore, the difference in the $^1O_2$-generation abilities of $LMIC_{60}$-**2** and $LMIC_{60}$-**3** could be attributed to differences in the amount of light absorbed by these materials at wavelengths greater than 620 nm (inset of Fig. 2a, red and green lines). These results suggested that the high photoactivity of C_{60}-**2** could be attributed to the introduction of a polar substituent rather than simply being associated with the derivatization of C_{60}. It is therefore very difficult to prepare non-polar C_{60} derivatives, as reference compounds with high photodynamic activities (e.g., C_{60}-**2**).

In the absence of a good reference compound, we compared the $^1O_2$ generating activity of *γ*-CDx with those the liposome incorporated C_{60} and its derivatives. Furthermore, given that C_{60} generates very little $^1O_2$ under photoirradiation at wavelengths greater than 620 nm, we employed light with a wavelength greater than 350 nm. As shown in Fig. 3d, the *γ*-CDx•C_{60} complex generated higher levels of $^1O_2$ than $LMIC_{60}$. In contrast, $LMIC_{60}$-**2** generated higher levels of $^1O_2$ than the *γ*-CDx•C_{60}-**2** complex. The contrasting results between C_{60} and C_{60}-**2** were attributed to (i) $LMIC_{60}$ quenching itself in the lipid membrane to a much greater extent than $LMIC_{60}$-**2** through self-aggregation (as indicated above in Fig. 2a); and (ii) $LMIC_{60}$ inhibiting energy transfer toward dissolved oxygen in the lipid membranes to a much greater extent than $LMIC_{60}$-**2**. Furthermore, the higher $^1O_2$ generating ability of $LMIC_{60}$-**2** compared with the *γ*-CDx•C_{60}-**2** complex suggests that C_{60}-**2** exists in close proximity to the surface of the lipid membrane. This result also indicates that photoexited C_{60}-**2** can readily interact with dissolved oxygen rather than self-aggregating in the same way as $LMIC_{60}$-**2**. In other words, although *γ*-CDx isolates C_{60}, it simultaneously protects photoexited C_{60} from the dissolved oxygen.[16] The location of the C_{60} derivatives in the lipid membrane is therefore one of most important factors for determining the efficiency of $^1O_2$ generation.

In summary, we have successfully prepared a series of $LMIC_{60}$ derivatives using an exchange reaction. The photodynamic activity of $LMIC_{60}$-**2** was much higher than that of $LMIC_{60}$, $LMIC_{60}$-**1** and $LMIC_{60}$-**3** and approximately two times higher than that of the porphyrin units of photofrin for the same photon flux (> 610 nm). The main reason for the high photodynamic activity of this material was attributed to the high $^1O_2$ generating ability of C_{60}-**2**. The location and isolation of C_{60}-**2** on the lipid membrane surface also played important roles in the photodynamic activity of this material compared with other fullerene derivatives. The latter of these two factors enhanced the generation of $^1O_2$ by $LMIC_{60}$-**2**. These findings could have important implications in biological, medicinal and materials chemistry applications involving the use of fullerene-based materials.

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14 We prepared $LMIC_{60}$, C_{60}-**1**, C_{60}-**2** and C_{60}-**3** with a cationic liposome surface using by a cationic lipid **5** ([**5**]/[**4** + **5**] = 10 mol%) since it has been known that $LMIC_{60}$ with the cationic liposome surface efficiently engenders cell death by photoirradiation, as compared with $LMIC_{60}$ with a neutral or an anionic liposome surface.[3a]

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