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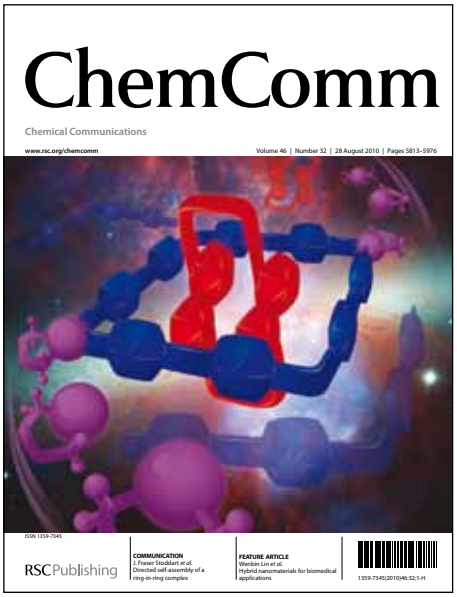

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COMMUNICATION

Dynamic behaviour in giant unilamellar vesicles induced by the uptake of [70]fullerene†

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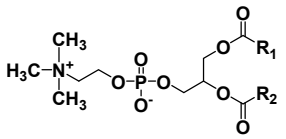
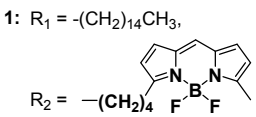
To obtain direct evidence for the fullerene-exchange reactions from the γ-cyclodextrin cavities to the lipid membranes following the addition of a C_{70}•γ-cyclodextrin complex, we monitored the dynamic behaviours of the giant unilamellar vesicles. A number of the C_{70} aggregates generated in the lipid membranes moved about vigorously.

In the last four decades, liposomes have received increasing levels of attention not only for their potential application as drug carriers but also as model cell membrane systems.[1] Fullerenes (C_x: x = 60 or 70)–incorporating liposomes ($LMIC_x$)[2,3] have been prepared by the fullerene-exchange reaction from the C_x•γ-cyclodextrin (γ-CDx) complex to small unilamellar vesicles (SUVs).[4-16] $LMIC_{60}$ prepared by the exchange method does not show any discernible morphological changes in its cryo-transmission electron microscopy (TEM) images ([C_{60}]/[dimyristoylphosphatidylcholine (DMPC)] = 30 mol%).[15,16] In contrast, in the cryo-TEM images of $LMIC_{70}$ ([C_{70}]/[DMPC] = 10 mol%), the majority of the liposomes exhibited humps in the lipid bilayer membranes. Furthermore, these humps become larger planar structures as the amount of C_{70} present in the system was increased ([C_{70}]/[DMPC] = 30 mol%).[16] These self-aggregates of C_{70} grow in a two dimensional manner. Investigations towards developing a deeper understanding of the behaviour of C_{70} incorporated in the lipid bilayer membrane are particularly important, not only in terms of the design of effective biomedical materials for applications using C_{70}, but also for identifying any of the potential deleterious effects of C_{70}.[17-20] In contrast, giant unilamellar vesicles (GUVs)[21] consisting of phospholipids have been used as models to study the membrane dynamics of living cells.[22,23] In the present study, we have performed a series of real-time observations of the dynamic behaviour in the fullerene-exchange process using phase contrast and fluorescence microscopies, paying particular attention to the aggregation of C_{70} within the lipid-membrane of GUVs.



POPC: R_1 = -$(CH_2)_{14}CH_3$, R_2 = -$(CH_2)_7CH{=}CH(CH_2)_7CH_3$
DMPC: $R_1 = R_2$ = -$(CH_2)_{12}CH_3$
DPPC: $R_1 = R_2$ = -$(CH_2)_{14}CH_3$



1: R_1 = -$(CH_2)_{14}CH_3$,
R_2 = —$(CH_2)_4$

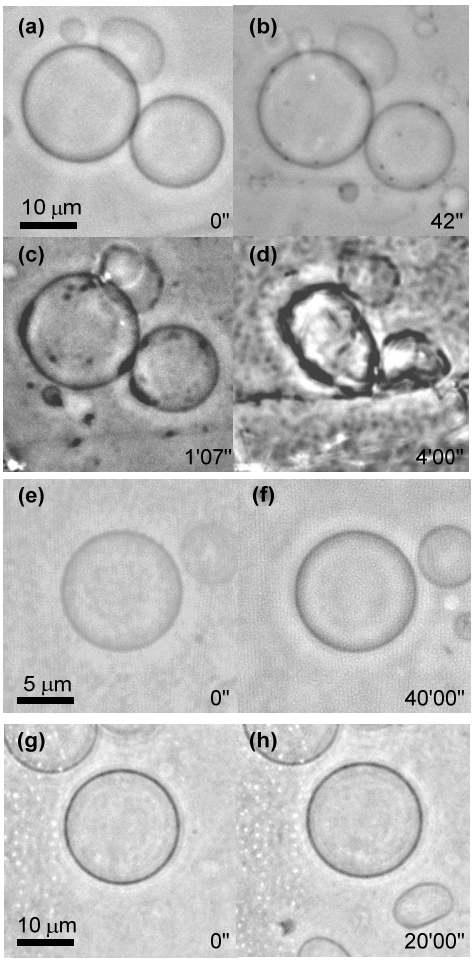


Fig. 1 Time-dependent shape transitions of the GUVs consisting of POPC following the addition of the (a)-(d) C_{70}•γ-CDx complex (0.25 mM) solution, (e) and (f) γ-CDx solution (1 mM) and (g) and (h) C_{60}•γ-CDx complex (0.25 mM) solution at ambient temperature. All of the micrographs are phase contrast images. The time elapsed following the starting injection of the C_{70}•γ-CDx complex solution or γ-CDx solution through the micropipette has been indicated for each image.

An aqueous solution of the C_{70}•γ-CDx complex (0.25 mM) was added to the GUVs consisting of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) and real-time phase

contrast images of the resulting mixture were measured using confocal laser microscopy (Figs. 1a-d and S1 and Movie S1). Following a period of 28 sec, although the shape of the GUVs hardly changed, large aggregates formed in the lipid membrane with no separation of the individual GUVs (Figs. 1b and S1b).[24] The number of aggregates increased over time, and the aggregates themselves were actively integrated through their movements into the lipid membrane (Figs. 1c and S1c and Movie S1). Finally, the integration of the aggregates led to the shrinkage of the GUVs (Figs. 1d and S1e). To confirm that the large aggregates consisted of C_{70} incorporated in the lipid membrane of the GUVs, the changes in the GUVs were investigated via the addition of an aqueous solution of γ-CDx alone (Figs. 1e,f and S2). In this particular case, no aggregation or changes in the shape of the GUV were observed, indicating that the addition of a large excess of γ-CDx alone ([γ-CDx] = 1 mM) could not change the morphology of the lipid membrane (Figs. 1f and S2b). Taken together, these results demonstrated that the large aggregates shown in Fig. 1b and 1c consisted of C_{70}. Furthermore, no changes in the shape of the GUVs in the γ-CDx alone case indicated that the shrinkage of the GUVs in the presence of C_{70} could be attributed not only to the osmotic pressure caused by the large excess of the C_{70}•γ-CDx complex ([total γ-CDx] = 1 mM) but also to the weakening of the vesicles of the GUVs by the integration of the C_{70} aggregates.

As shown in Figs. 1g, 1h and S3, the addition of the C_{60}•γ-CDx complex at ambient temperature did not lead to the aggregation or change in shape of the GUVs consisting of POPC.[25] Two potential explanations have been provided to explain why no C_{60}-aggregates formed in this experiment, including (i) the C_{60}-aggregates in the lipid membrane were too small to be observed by phase contrast microscopy, and (ii) C_{60} was not incorporated into the liposomes. To determine which of these explanations was correct, we collected fluorescence microscopy images using the GUVs consisting of POPC and 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (**1**) ([**1**]/[POPC] = 0.25 mol%). When **1** and C_{60} or C_{70} coexisted in the liposomes, the C_{60} or C_{70} acted as a quencher. The addition of the C_{70}•γ-CDx complex led to the immediate quenching of the fluorescence (Fig. S4a and S4b), whereas the addition of the C_{60}•γ-CDx complex did not, even after 40 min (Fig. S4c-f). These findings strongly supported the suggestion that C_{60} was not incorporated into the liposomes [i.e., explanation (ii)] and are consistent with the previous result that $LMIC_{60}$ cannot be prepared by the exchange method at ambient temperature.[6]

We recently reported that the occurrence of a complete C_{70}-exchange reaction at temperatures above the phase transition temperature (T_m) between the gel- and liquid crystal-phases of the SUVs. Interestingly, however, when the temperature was lowered to a value below the T_m, barely any of the C_{70} was transfer from the γ-CDx cavity to the membranes of the SUVs.[12,13] To investigate the effect of the T_m of the GUVs on the aggregation process, the C_{70}-exchange reaction was conducted at temperatures above and below the T_m value of using GUVs composed of dipalmitoylphosphatidylcholine (DPPC, T_m = 41°C) and DMPC (T_m = 23°C). No shape transitions occurred in the GUV composed of DPPC following the addition of the C_{70}•γ-CDx complex at ambient temperature ($<T_m$), even after 20 min (Figs. 2a, 2b and S5). Fluorescence microscopy of the GUVs consisting of DPPC and **1** ([**1**]/[DPPC] = 0.25 mol%) revealed that the

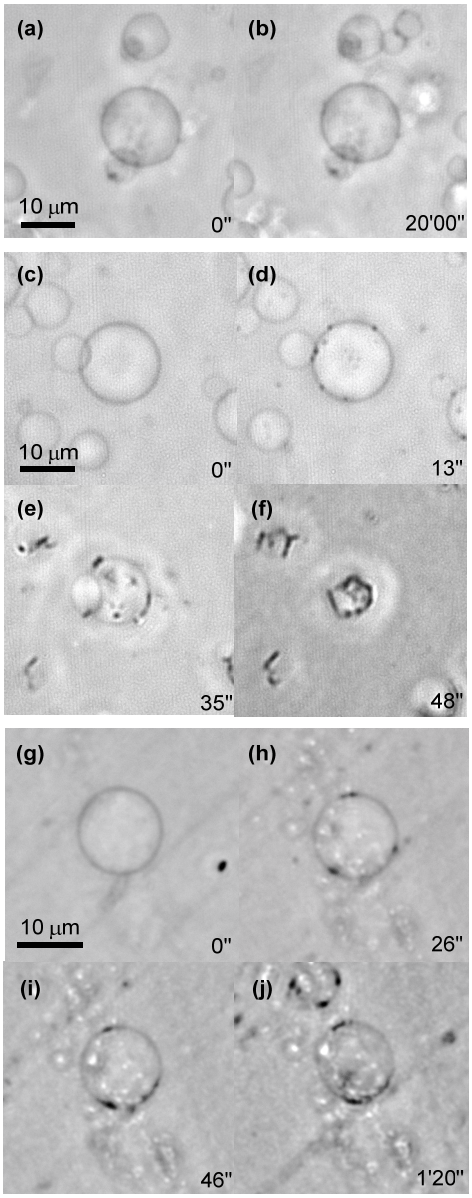


Fig. 2 Time-dependent shape transitions of the GUVs consisting of (a) and (b) DPPC following the addition of the C_{70}•γ-CDx complex solution at ambient temperature, (c)-(f) DMPC following the addition of the C_{70}•γ-CDx complex solution at 37°C, and (g)-(j) DPPC following the addition of the C_{70}•γ-CDx complex solution at 44°C. All of the micrographs are phase-contrast images. The time elapsed following the injection of the C_{70}•γ-CDx complex solution through the micropipette has been indicated for each image.

addition of the C_{70}•γ-CDx complex did not lead to fluorescence quenching (Fig. S6), indicating that the C_{70} had not been incorporated into the liposomes. To confirm whether the observed differences in behaviour between the POPC and DPPC were caused by the effect of T_m or by the structures themselves, DMPC with a structure similar to that of DPPC was also used (T_m = 23°C). The GUVs consisting of DMPC at 37°C ($>T_m$) behaved in a similar manner to the GUVs consisting of POPC (T_m<0°C) at

ambient temperature (Figs. 2e, 2f and S7), suggesting that the different behaviours observed at ambient temperature were related to the T_m values of the materials. The conclusion was further supported by the observation that the behaviour in the GUVs consisting of DPPC at 44°C ($>T_m$) was similar to that of the GUVs consisting of POPC at ambient temperature (Figs. 2g-j and S8). These results indicated that the C_{70}-exchange reaction only occurred at temperatures greater than the T_m, which was in agreement with our previous results in the SUVs.[12,13]

Attempts to observe changes in the shape of the GUVs following the C_{70}-exchange reaction at higher temperatures failed because the GUVs were not stable, and heating at temperatures in excess of 50°C was required to evaporate the solvent. We therefore investigated the influence of the addition of the C_{70}•γ-CDx complex at 80°C using cryo-TEM images of the SUVs consisting of DMPC. As shown in Fig. 3a, no aggregation or changes in the shape of the SUVs were observed for the exchange reaction when it was conducted at 80°C. Interestingly, however, small aggregates of C_{70}, although unobservable in the cryo-TEM images, were formed in the lipid membranes based on the peak broadening of the absorption band (Fig. S9). In a separate experiment, when the solution was heated at 80°C for 2 h following an exchange reaction at 30°C, humps remained on the surfaces of the liposomes (Fig. 3b), indicating that the formation of the large C_{70}-aggregates was an irreversible process and that the large aggregates did not grow in the C_{70}-exchange process at 80°C.

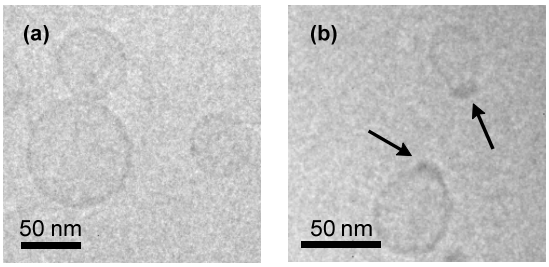


Fig. 3 Cryo-TEM images of SUVs consisting of DMPC following the addition of the C_{70}•γ-CDx complex solution (a) at 80°C, and (b) at 30°C before being heated at 80°C for 2 h ([C_{70}]/[DMPC]= 10 mol%).

In summary, we have directly observed the processes involved in a C_{70}-exchange reaction using GUVs. At temperatures greater than the T_m, the C_{70}-aggregates migrated into the lipid membranes and became progressively larger through coalescence with other similar aggregates. Finally, the resulting huge aggregates led to the shrinkage and, in some cases, occasional bursting of the GUVs. At temperatures below the T_m of DPPC, the GUVs did not change in shape and no C_{70} aggregates were observed in the membrane. These results were consistent with our previous findings, where the C_{70}-exchange reaction was found to be dependent on the T_m of the liposomes in the SUVs. No shape transitions or aggregation events were observed in the SUVs when the C_{70}-exchange reaction was carried out at 80°C. Given that the C_{70}-exchange reaction occurred at this temperature, it has become apparent that large aggregates were not generated at the high temperature. The observation of this behaviour in the artificial lipid membrane could be very important not only in terms of the design of effective biomedical materials for use in photosensitisers in photodynamic therapy using C_{70} but also for identifying any potential deleterious effects of C_{70} in human cells.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [Experimental procedures, movie of phase contrast microscopy, phase contrast and fluorescence microscopy images and UV-vis absorption spectra]. See DOI: 10.1039/b000000x/

‡ This papre is dedicated to the 70th birthday of Professor S. Shinkai.

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24 Delay times until appearances of aggregations on the GUV surfaces were unsettled because each sample had a different distance between the GUV and the tip of micropipette.
25 The C_{60}-exchange reaction from a γ-CDx cavity to liposomes hardly occurs at room temperature.