

Supporting information

Transmission of photo-catalytic function in a self-replicating chemical system: in-situ amphiphile production over two protocell generations A. N. Albertsen,^a S. E. Maurer^b, K.A. Nielsen^{a,c} and P.-A. Monnard^a

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General description of the photochemical amphiphile production

The photochemical conversion of precursor lipid, pL, into decanoic acid (DA) to grow and divide the bilayers anchored to the microspheres was carried out using a catalytic system derived from that described by DeClue *et al*^{1,2}. This original system was composed of ruthenium trisbipyridine (as photosensitizer and catalyst), 8-oxoguanine (electron donor for the quenching of the first ruthenium complex excited state) and 1,4-dihydrophenylglycine (as hydrogen donor and electron source during the cleavage of the picolylium and regeneration of 8-oxo-guanine).

The pL conversion in this paper was dependent on a lipophilic ruthenium complex, Figure SA, as a photocatalyst that is excited by visible light. Furthermore, 1,4-dihydrophenylglycine and 8-oxoguanine were replaced by ascorbic acid to reduce the catalyst sensitivity to oxygen and increase the reaction rates. Ascorbic acid acted as both electron donor for the reductive quenching of the ruthenium complex excited state, Figure SB-A, and hydrogen donor during the cleavage of the pL ester bond, Figure SC. Even though ascorbic acid significantly reduces oxygen sensitivity, the amount of oxygen was still minimized by sonicating the solutions under Argon.

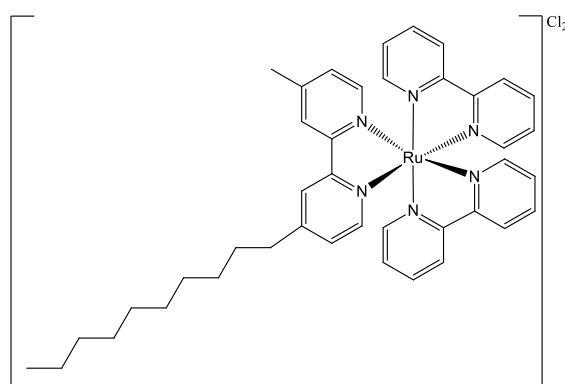


Figure SA. Chemical structure of the lipophilic ruthenium complex used in the ascorbic acid based metabolism Bis-(2,2'-bipyridyl) (4-decyl,4'-methyl-bpy)ruthenium(II) chloride (RuC10:0).

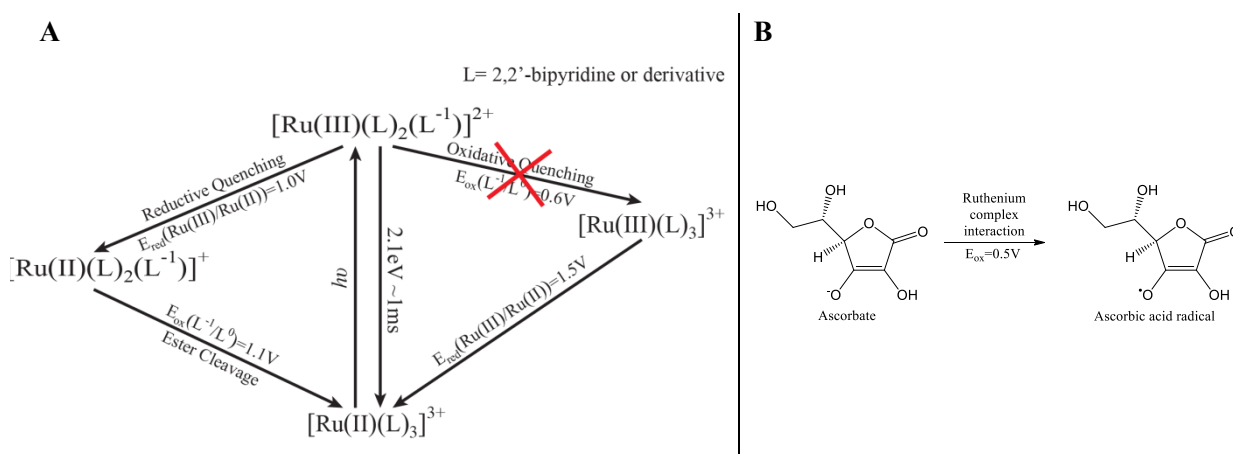


Figure SB. (A) The photochemical cycle of the ruthenium complex during the reaction³. The system is designed to proceed via a reductive quenching pathway from the excited state MLCT of ruthenium due to the E_{ox} which is necessary to cleave pL. (B) The pathway for reductive quenching of the ruthenium complex by the ascorbic acid during the photochemical conversion.

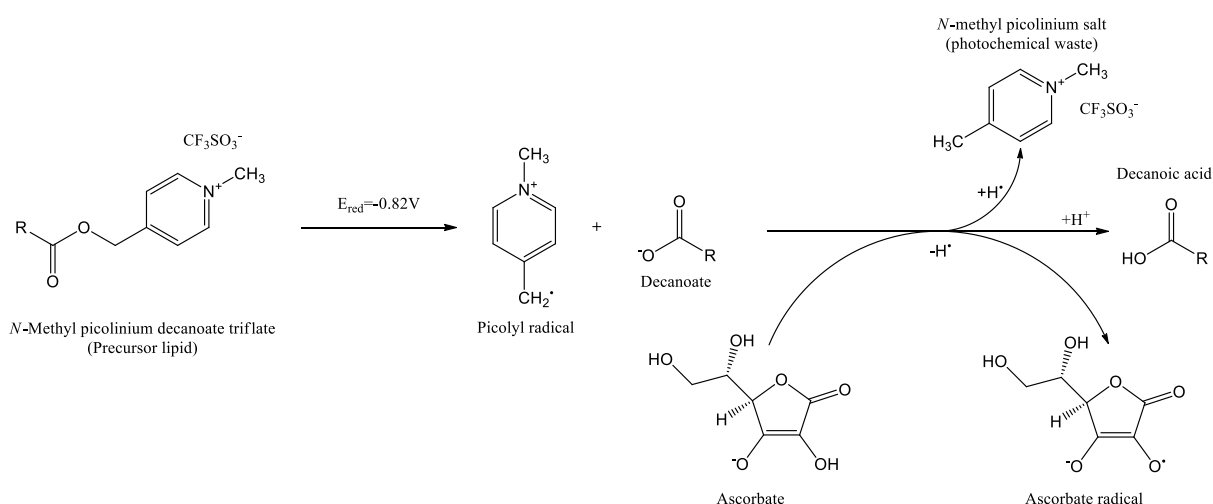


Figure SC. The photochemical cleavage of the pL to *N*-methyl picolinium salt and decanoic acid (DA) at the expense of an ascorbic acid molecule. $R = CH_2(CH_2)_7CH_3$.

Both of these photocatalytic systems were capable of converting pL into DA when illuminated by visible light. But, the reaction rates were faster in the case of the ascorbic acid system because its higher water solubility allowed for higher concentrations.

In a competing side reaction, the ester bond in the pL is slowly hydrolysed at pH 7. The hydrolysis reaction also produces decanoic acid (DA), however the reaction results in a different cleavage product called hydrolysis waste product, Figure SD. The hydrolysis waste product can be separated from the photochemical waste product, thereby independently quantified by HPLC analysis.

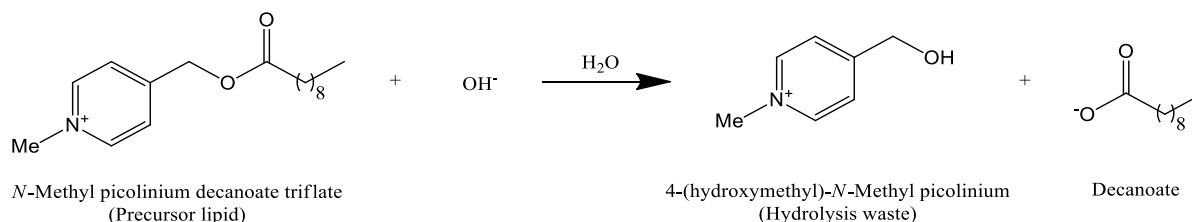


Figure SD. The hydrolysis of the pL. The hydrolysis results in a DA molecule and the hydrolysis waste product.

In order to determine the primary pathway for the synthesis of decanoic acid, all reacted samples were analysed by HPLC, which allows us to establish the ratio between the photochemical waste product and the hydrolysis waste product. This ratio revealed which of the two competing reaction pathways was the predominant during the experiment.

2. General description of the protocell

The protocell concept presented in this study is based on the amphiphile bilayers themselves as the center of protocell's chemistry, its compartment, as opposed to the aqueous lumen contained within bilayers. This particular embodiment of the protocell compartment/catalytic network remedies some issues (e.g., encapsulation yields, permeability to charged solutes) encountered in the more commonly envisioned containers that consist of the aqueous lumen within bilayer vesicles. Indeed, decanoic acid-decanoate vesicles have been shown to release their aqueous content even at 20 °C^{4,5}. Inserting the catalytic system within the bilayers can more extensively preserve the integrity of the container-metabolism assembly, thereby the protocell functionality.

The localization of a simple metabolism in the boundary structure itself also facilitates the access to substrates with a hydrophobic moiety, such as the amphiphile ester precursor, as they will spontaneously associate with the container. As a corollary, water-soluble waste products that might poison a reaction will be released and diluted into the large, external, aqueous volume. However, it should be noted that the type and complexity of the catalytic assemblies anchored in vesicle bilayers will be limited compared to the complexity of a transcription-translation apparatus sometimes encapsulated in aqueous lumina of vesicular and liposomal protocell models^{6,7}. In addition, fully stable fatty acid vesicles might be still necessary to achieve a differentiated reproduction of an individual protocell at a later stage of the protocell evolution. Namely to apply proper selection pressure, reproduction needs to occur predominantly through production of the protocell's own components rather than merger of protocells.

The strategy followed to construct the glass microsphere-supported protocell starts with the coating of the microsphere with avidin, which followed by the incubation with biotylated lipids. The decanoic acid bilayers are deposited on the derivatized microspheres and the molecules composing the photocatalytic system are then added. Between each preparation step, the excess of the chemicals is removed by a series of washes. To start the photochemically driven replication of the bilayers, the amphiphile precursors are slowly injected while the samples are irradiated in the presence of ascorbic acid or other compounds that serve as electron relay or radical scavenging species, Figure SE.

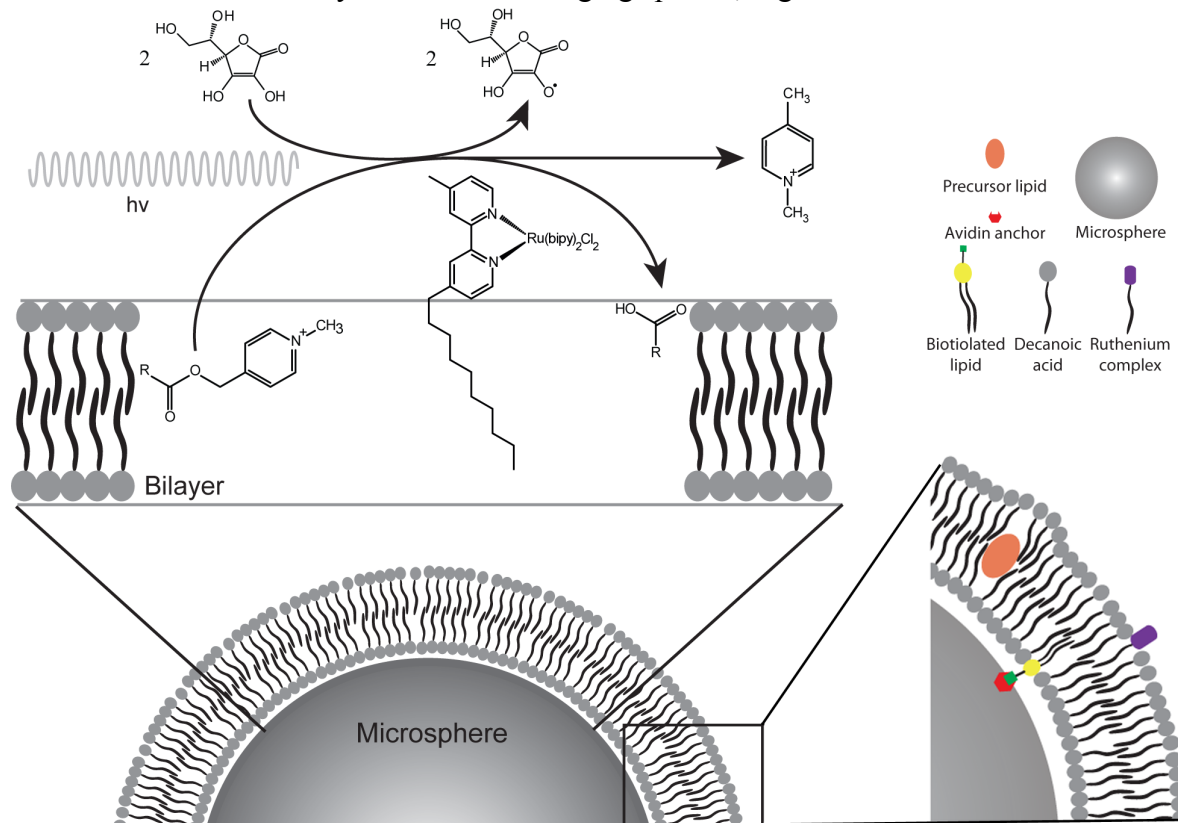


Figure SE. Schematic representation of the protocell. On top, the photochemistry that occurs with the bilayers. On the right, the chemical details of the protocell. For decanoic acid molecules, R = (CH₂)₈CH₃.

The construction of the protocell is therefore based on non-covalent interactions: association between the glass surface and avidin, bio-affinity between avidin and biotin and the balance between the hydrophobic/hydrophilic properties of all other compounds.

3. Materials

All chemicals were of the highest purity possible and bought from Sigma-Aldrich, Schnelldorf, Germany unless otherwise noted. All chemicals were used as received and no further purification was performed. Decanoic acid (DA) 99 % was purchased from Acros Organics (Geel, Belgium). 1,2-disteroyl-*sn*-glycero-3-phosphoethanolamine-N-[biotinyl(polyethyleneglycol)2000] ammonium salt (biotinylated lipid) was bought from Avanti Polar Lipids, Inc. Alabaster, Alabama, USA. Fluorescently labelled lipids, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) ammonium salt (NBD-PE) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt (Rh-DHPE) were purchased from Avanti Polar Lipids.

The water used was of Milli-Q grade, 18.2 M Ω ·cm at 25°C with 3 ppb TOC.

The glass microspheres were ordered from Bang Laboratories Inc., Indiana, USA. The microsphere dispersion from the supplier was sonicated (bath sonicator, SONOREX DIGITEC from Bandelin electronic, Berlin, Germany) for one minute prior to use, to re-disperse the microspheres. 20.76 μ L were dispersed in 1980 μ L of water yielding $9 \cdot 10^6$ glass microspheres per mL.

Throughout all experiments “buffer” refers to an aqueous solution containing 100 mM of monobasic sodium phosphate adjusted to pH 7.1. In all other instances, the pH and concentration of buffering agent is specified. The hosting and washing solutions consisted of buffer (100 mM of phosphate buffer at pH 7.1) unless otherwise noted.

The following compounds were synthesised according to literature syntheses: N-Methyl picolinium decanoate triflate (pL or precursor lipid) according to¹ and bis-(2,2'-bipyridyl)-(9-N-[4-(4'-decyl-[2,2']bipyridinyl-5-yl)-butyl]-8-oxoguanine)ruthenium(II) chloride (RuC10:0 oxoG) and Bis-(2,2'-bipyridyl) (4-decyl,4'-methyl-bpy)ruthenium(II) chloride (RuC10:0) both according to².

4. Experimental Procedures

Direct DA coating of microspheres (without avidin)

A DA vesicle suspension (50 mM DA in buffer) was prepared using the titration method described by Hargraves *et al.*⁸. First, the microspheres were transferred into 1 mL buffer. A 200 μ L aliquot of this dispersion was taken, centrifuged (Heraeus Biofuge pico, Kendro Laboratory Products GmbH, Hanau, Germany) for 20 minutes at 6000 RPM (3421 RCF). Its hosting solution was replaced by 200 μ L of the decanoic acid vesicle suspension. The resulting dispersion was placed on a shaker at 500 RPM for 10 minutes, sonicated for 10 minutes, and incubated overnight at 30 °C.

The microsphere dispersion with DA was washed with buffer. The resulting microspheres were analysed on the microscope.

Preparation of the avidin coated glass microspheres

Each sample was vortexed for one minute and sonicated for 10 minutes. The sample was then centrifuged for 20 min. The supernatant (the hosting solution) was removed and the microsphere pellet was re-dispersed in 1982 μ L of buffer, to which 18.18 μ L of 0.5 mg/mL avidin solution was added. The dispersion was vortexed for one minute and left at 5 °C overnight.

To remove the excess of avidin from the dispersion, three cycles of centrifugation and re-dispersion in buffer were performed. The avidin covered microspheres ($9 \cdot 10^6$ glass microspheres per mL) were placed in a storage solution containing buffer.

Anchoring of a DA bilayer to the glass microspheres

Biotinylated lipid in chloroform (18 μL 1 mg/mL) were mixed with 42.9 μL 1.1 M DA in methanol and the mixture was dried under vacuum overnight. The resulting lipid film was re-suspended in a solution containing 250 μL 200 mM phosphate buffer at pH 9.2, 200 μL water, and 20 μL 2M NaOH. Vesicles were then formed using a pH titration procedure, as described by Hargreaves *et al.*⁸. The final suspension contained 118 mM DA and 14.9 μM biotinylated lipid.

Equal volumes of vesicle suspension and avidin covered microspheres were mixed on a shaker at 500 RPM for 10 minutes (IKA Vibrax VXR basic from IKA Works, Inc. Wilmington, North Carolina, USA), then sonicated for 2 minutes, and equilibrated for 10 minutes at room temperature. The incubation solution was removed and the microspheres were re-suspended in 400 μL buffer. The solution hosting the microspheres was exchanged twice to remove the excess of DA (centrifugation for 20 min at 6000 RPM). The bilayer-coated microspheres ($9 \cdot 10^6$ microspheres/mL) were stored in buffer.

Determination of the DA concentration

To quantify the amount of fatty acid in the solution and the amount of fatty acid associated with the microspheres, DA was derivatized using the method described by Durst *et al.*⁹ for UV detection on the HPLC. DA was extracted from the various aqueous phases and the microspheres, dried down and then resuspended in 300 μL acetonitrile. An additional 300 μL acetonitrile containing 75 mM (0.0225 mmole) triethylamine and 25 mM (0.0075 mmole) 4-bromophenacyl bromide were then added. The samples were vortexed, heated to 60°C for 30 minutes and diluted to 1 mL with acetonitrile, before injection on the HPLC. The concentration was then determined using a calibration curve prepared the same way.

The hosting solutions were analysed directly after separation from the microspheres, whereas the quantification of fatty acid composing anchored bilayer required the removal of the microspheres before analysis. The bilayer was removed by increasing the pH of the final hosting solution with the addition of 10 μL of 2 M NaOH. The amount of HCl used at the beginning of the derivatization process was adjusted accordingly.

After the total deprotonation of the fatty acid (by addition of NaOH), the hosting solution was removed from the microspheres by centrifugation. The fatty acid in the hosting solution was then re-protonated by lowering the pH to approximately 1, and the protonated species were then extracted with two volume equivalents of CHCl_3 . The CHCl_3 phase was then evaporated. The dried DA was finally dissolved in ACN to carry out their derivatization.

Samples (100 μL) were then analysed by HPLC (Agilent Technologies 1200 series HPLC with UV/Vis detector). Using an Agilent Poroshell 120 C-18 4.6 x 150 mm, 2.7 μm column at ambient temperature, and a flow rate of 0.5 mL pr minute the following gradient was employed starting with 0.1 % v/v trifluoroacetic acid (TFA) in Milli-Q water water and 20 % methanol for 2 min. The gradient was increased to 100 % methanol from 2-17 minutes and held at 100 % methanol until 22 min. The gradient was then returned to the starting mobile phase (20 % methanol) from 22-31 min. and held until 34 min to re-equilibrate the column. UV-Vis measurements were made at 254 nm.

Interaction between the ruthenium complex and the anchored bilayer

The microsphere hosting solution was exchanged once with buffer, before being re-suspended in a ruthenium solution containing 5 mM RuC10:0 in buffer. The microspheres were incubated for 1 h under shaking (500 RPM) to ensure mixing and prevent sedimentation.

The non-associated ruthenium was then removed by exchanging three times (centrifugation) the solution with buffer, and incubating the microspheres under shaking for 2, 3, and 24 h. After separation from the microspheres, the amount of ruthenium complex in the washes was

quantified by fluorescence spectrometry using the intrinsic fluorescence of RuC10:0, see below.

The microspheres were finally re-suspended in 200 μ L buffer to which 10 μ L 10 % w/w Triton X-100 were added. The resulting dispersion was sonicated for 2 minutes and placed on the shaker at 500 RPM overnight, as above the microspheres were removed from the solution and the amount of ruthenium once associated with the microsphere was analysed.

Specific calibration curves with the different concentration ranges were compiled from serial dilution of ruthenium complex solutions to account for self-quenching at concentrations above 0.208 mM and nonlinear emission below 0.052 mM. These calibration curves enabled the measurement of the ruthenium concentration in the various washes and microsphere dispersions. The fluorescence signals were measured on a Varian Cary Eclipse Fluorescence Spectrophotometer. Varian, Inc, Palo Alto, California, USA. The samples were excited at 450 nm and the emission was measured at 620 nm.

Interaction between vesicles and the ruthenium complex in the anchored bilayer

A dispersion containing microspheres with anchored bilayers was incubated with 5 mM RuC10:0 and washed three times to remove any ruthenium complex not associated with the anchored bilayer. After the final wash, the ruthenium complex associated to DA coated microspheres was tested for strength of the association. This was done by re-suspending the microspheres in a solution containing DA vesicles prepared by pH titration (20 mM DA in buffer). The mixture was incubated on a shaker at 500 RPM for 24 hours at room temperature, before the hosting solution, containing the vesicles, was removed from the microspheres by centrifugation. To destroy the vesicles, 7.5 μ L 10 % w/w Triton X-100 was added to the hosting solution before fluorescence analysis (see above). The bilayers were removed from the microspheres by addition of 7.5 μ L 10 % w/w Triton X-100 to the solution and subsequent sedimentation by centrifugation before fluorescence analysis.

Rate of insertion of precursor into preformed bilayers

Films were prepared to contain 25 mM DA, 0.03 mM NBD-PE, and 0.03 mM Rh-DHPE when suspended in buffer, by the thin film method. Briefly, neat lipids were dissolved in chloroform at known concentrations. The lipid/chloroform solutions were then mixed to give the concentrations specified above, and dried under argon flow. Remaining chloroform was removed by placing the samples under vacuum for < 2 h. Samples were kept from light during preparation.

To determine the insertion of pL, a standard curve was prepared from the same stock solutions of lipid/chloroform to ensure accurate concentrations. In addition to the 25 mM of DA and 0.03 mM of FRET markers, pL was added to the film preparation, dissolved in chloroform, in concentrations ranging from 2 mM to 25 mM.

Films were rehydrated in phosphate buffer and vesicles formed using a pH titration procedure, as described by Hargreaves *et al.*⁸ (final concentration: 25 mM DA, 0.03 mM NBD-PE, and 0.03 mM Rh-DHPE, 100 mM phosphate buffer pH 7.2). Samples were mixed using a vortex for several minutes then sonicated in a bath sonicator for 30 minutes at room temperature to ensure suspension of the lipids.

The FRET marker/DA vesicles were placed into the fluorimeter with a small magnetic stir bar (1 cm square cuvette). Fluorescence values were read every 30 seconds with excitation at 470 nm and emission at both 515 and 605 nm to monitor the donor and acceptor molecules simultaneously. pL (200 mM) dissolved in ethanol was injected at a rate of 30 μ L/h using a syringe pump (TSE systems Programmable Syringe Pump model no. 540060. TSE systems GmbH, Bad Homburg, Germany). Once the concentration of pL in the reaction cuvette reached 12 mM the injection was stopped, but measurements were continued until the signal stabilized.

The insertion of pL was also monitored in supported bilayers. 5 μL 500 mM pL in 96 % ethanol was slow injected to 500 μL of microspheres with anchored DA bilayers (see above) at a 3.33 $\mu\text{L}/\text{h}$ rate, using a 10 μL Hamilton syringe and the syringe pump. During the slow injection, the microsphere dispersion was sampled at intervals corresponding to 0.75 μL of injected pL. These samples were stained with Nile red and investigated on the microscope.

Microscopy of pL in DA vesicles

A 30 mM DA vesicle suspension was prepared by pH titration, final pH 7.2 and containing 100 mM of phosphate buffer. Before slow injection of pL the vesicles were extruded to 400 nm. 3.75 μL of the pL solution, 500 mM in ethanol, was injected at a rate of 1.87 $\mu\text{L}/\text{h}$ to 500 μL of vesicle solution. The mixture was sampled after the injection of 0, 0.375, 0.750, 1.125, 1.500, 1.875, 2.250, 2.625, 3.000, 3.375 and 3.750 μL of pL. During the injection, the sample was maintained at 25°C, before microscope investigation the samples were stained with Nile red.

Photochemical conversion of 16.2 mM pL using ascorbic acid

Avidin-covered microspheres were prepared as described above and re-suspended in a suspension containing the amphiphiles, pL, the ruthenium complex (94.37 mM DA, 14.9 μM biotinylated lipid, 16.2 mM pL 0.5 mM RuC10:0). The new dispersion was placed 10 minutes on a shaker (500 RPM), 2 min in the sonicator, and incubated for 10 minutes at room temperature. The non-associated ruthenium was then removed by washing the microsphere pellets three times with buffer.

The irradiation solution contained 16.2 mM pL and 48 mM ascorbic acid in buffer. The sample was degassed under argon flow for 30 minutes on the sonicator before being mixed with the bilayer anchored microspheres.

Once mixed, the samples were irradiated for 24 hours using a 1 mm path length cuvette and a cold light source with a fibre optic bundle attached to the cold light source with 150 W halogen bulb set to obtain a 3000 K, black body, spectrum (KL1500 LCD, Leica Microsystems AG, Heerbrugg, Switzerland). The incubation temperature was maintained at 25 °C using a heating block.

After irradiation, the reaction solution was separated from the microspheres by centrifugation. Finally, the microspheres were washed with 400 μL methanol to remove remaining lipids. The supernatant and the methanol wash were analysed by HPLC.

Photochemical conversion using ascorbic acid and slow injection

The microsphere systems, with anchored bilayers, were prepared as described above. Once the bilayers were anchored to the microspheres the ruthenium complex was added (10 μM) to the sample, the pL was slowly injected into the irradiated sample. The final composition of the samples was 10 μM ruthenium, 0.421 mM DA, and 30 mM ascorbic acid, corresponding to a 47:1 ratio of decanoic acid and ruthenium.

The pL solution (500 mM in ethanol) was injected into the reaction cuvette, the cuvette was connected to the syringe pump and the sample was illuminated using a same set-up as described above. Over the course of the first 48 hours, of the experiment, the pump injected 9.6 μL of pL solution at a rate of 0.2 $\mu\text{L}/\text{h}$. The reaction sample was placed in a 1 mm pathlength cuvette and Leica KL1500 LCD cold light source (Leica Microsystems AG, Heerbrugg, Switzerland) with a fibre optic bundle attached to the cold light source (150 W halogen bulb set to obtain a 3000 K spectrum) was used to illuminate the sample. A heat block maintained the temperature at 25°C for the duration of the experiment (168 h).

After irradiation, the reaction solution was separated from the microspheres by centrifugation. Finally, the microspheres were washed with 400 μL methanol to remove remaining lipids. The supernatant and the methanol wash were analysed by HPLC.

Continued photochemical conversion by the microspheres and vesicles

The microspheres were re-suspended in a solution containing 3 mM pL and 6 mM ascorbic acid in buffer. Prior to mixing with the microspheres, the solution was degassed with argon under sonication for 30 minutes. At the end of the irradiation (168 h), the hosting solution was removed from the microspheres, the microspheres were washed with 400 μ L of methanol. The hosting solution and the methanol wash was analysed by HPLC.

To 350 μ L supernatant from the initial experiment reaction dispersion (it contained the newly formed vesicles), 12 mM pL and 24 mM ascorbic acid were added, the resulting mixture was degassed under argon for 30 minutes on the sonicator. The degassed mixture was placed in a cuvette and illuminated for 72 h at 25°C. At the end of the irradiation period, the sample was analysed by HPLC.

Photochemical conversion using 8-oxoguanine

In this version of the experiments, the microspheres with anchored bilayers were prepared as described previously described. The bilayers were mixed with 200 μ L 20 μ M 8-oxoG-Ru10C and 17 mM DHPG in buffer, the solution was left on the shaker for 15 minutes at 500 rpm. To this dispersion, 12 mM pL and 17 mM DHPG in buffer were added. The combined 400 μ L was then degassed under argon flow while being sonication for 30 minutes in the dark.

The solution was irradiated for 120 hours, while the temperature was maintained at 25 °C by keeping the side opposite the reaction cuvette in contact with a heating block. At the end of the irradiation, the hosting solution was removed from the microspheres and the microspheres were washed with 400 μ L of methanol. The hosting solution and the methanol wash was analysed on the HPLC.

HPLC analysis of precursor and derivatives

The samples were run on an Acclaim 120 column (250 x 4.6 mm, C-18 silica, Dionex). Solvent A was 0.5 % trifluoroacetic acid in water by volume and Solvent B was 90 % methanol and 10 % Solvent A by volume. The solvent flow was at a rate of 0.75 mL per minute with the following gradient: 0 to 2 min at 100 % A, from 2 to 27 min increase from 0 % to 37 % B, from 27 to 37 min the gradient was increased from 37 % to 77 % solvent B, from 37 to 52 min increase 77 % to 100 % B, from 52 to 60 min 100 % B was used, followed by equilibration with solvent A for 5 min. UV-Vis measurements were made at 254 nm. This wavelength corresponds to the maximum of the absorption band of the N-methylated picolinium ester ring system and the ruthenium bipyridine absorption spectra, respectively.

The position of the various components was monitored by co-injection of standards: 10 min, picolinium decanoate hydrolysis product (4-(hydroxymethyl)-1-methylpyridinium); 16.7 min, precursor waste product (1,4-dimethylpyridinium), 47 min, pL as described in². Before injection the samples were diluted four times with 1 % Triton X 100 solution, if the sample contained microspheres, they were removed by centrifugation prior to injection.

Microscopy procedure

Epifluorescence microscopy was carried out on a Nikon TE2000-S microscope, Nikon Japan, connected to BD CARV II, NJ USA, using a Photometrics Cascade II 512 camera, Tucson Arizona USA. The samples were illuminated with a Nikon Intensilight C-HGFI, Nikon Japan, fluorescent lamp. Both microscope slide and cover slip were treated with PlusOne Repel-Silane ES (GE HealthCare Life Sciences, Little Chalfont, United Kingdom). A spacer was used between microscope slip and slide in all samples containing microspheres, to make room for the microspheres between the two glass sheets.

To visualize the samples they were stained with a volume of 1 mM Nile red in ethanol corresponding to maximally one tenth of the sample volume, unless otherwise noted. The exposure was set to 50 ms unless otherwise noted.

5. Results and discussion

5.a: Anchoring of DA bilayers on glass microspheres

Two anchoring procedures were employed to associate DA bilayers with glass microspheres. The first simply mixed the amphiphiles with glass beads¹⁰. The association was dependent on the interactions between the positively charged glass surface and the negatively charged decanoate headgroups. The second procedure, which was designed for this study, was adapted from a procedure described by Gopalakrishnan *et al.*¹¹ for phospholipid bilayers anchoring on beads. In this case, the bilayers were tethered on the glass surface taking advantage of the strong interactions between biotinylated lipids (modified phospholipids) and avidin coating the microsphere surface.

The success of each preparation procedure was investigated using epifluorescence microscopy and the spectroscopic determination of the concentration of derivatised DA. The microspheres were visible by Nile red fluorescence when DA bilayers were associated with them because of hydrophobic interactions between the dye and the DA (Figure SF, micrographs 1 & 2 versus 3 & 4).

The first coating procedure resulted in bilayer-covered microspheres. However these bilayers were not resistant to exchanges of hosting solution and would easily diffuse away from the microsphere surface. Exchanging the hosting solution once resulted in a decrease in the signal to noise ratio (Figure SF micrographs 5 and 6).

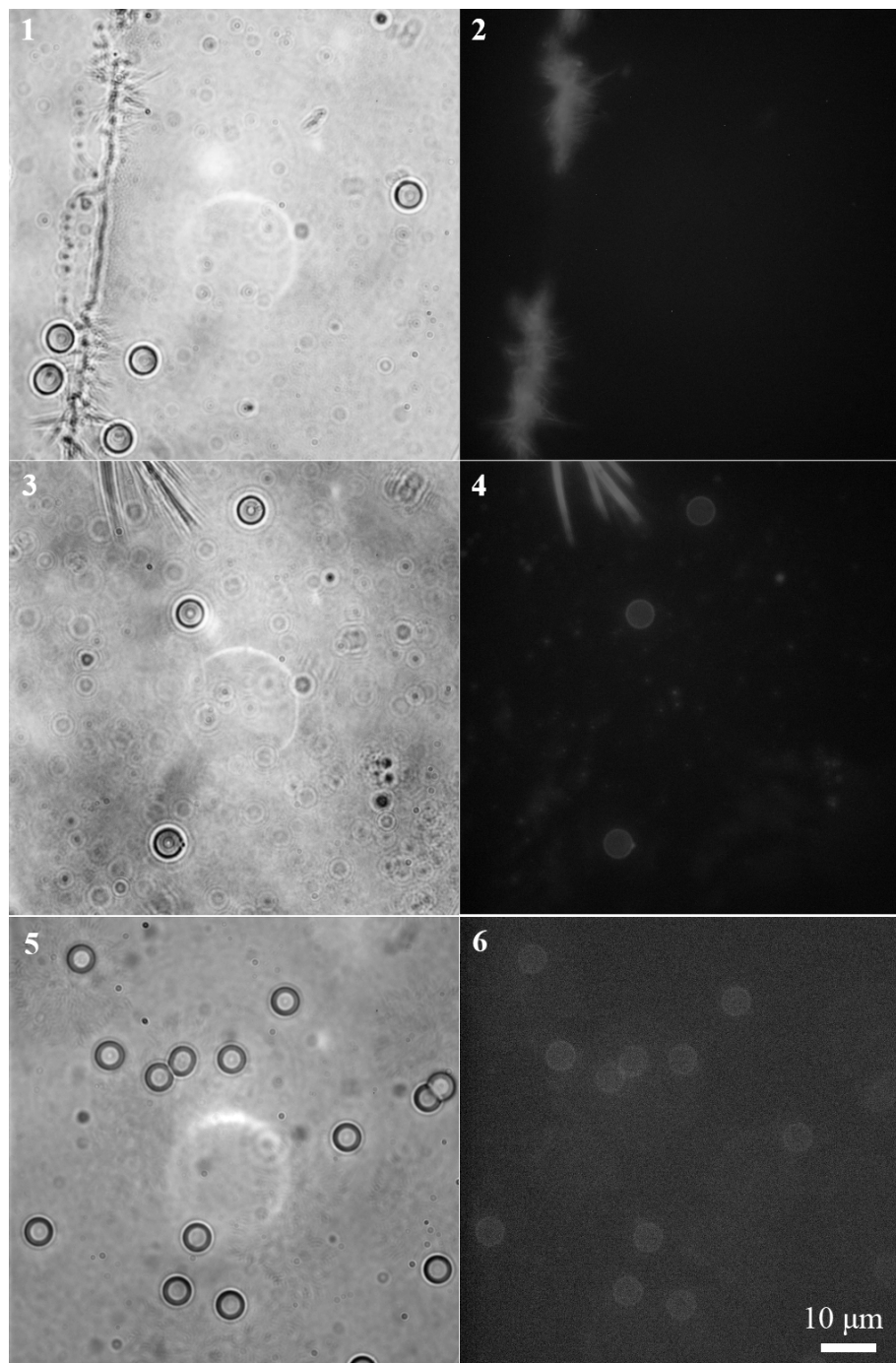


Figure SF. DA coating of glass microspheres. Odd numbered and even numbered micrographs were taken using bright field microscopy or the fluorescent signal of Nile red, respectively. All samples were stained with 0.1 mM of Nile red. 1 & 2 micrographs of avidin coated microspheres with no DA bilayers. 3 & 4 displays microspheres coated with DA bilayers (second procedure), after exchanging the hosting solution three times. 5 & 6 microspheres coated with DA (first procedure) after exchanging the hosting solution once.

The decrease in the signal to noise ratio indicated that some of the bilayers were removed by exchanging the hosting solution. The failure to retain the bilayers during exchange made the first procedure unsuitable for the study of growth and division of protocellular systems.

Using avidin/biotin linkages to anchor lipids to the microspheres resulted in associated bilayers, which were resistant to wash. As demonstrated in Figure SG, it was possible to reduce the fatty acid concentration in the washes to below the detection limit of the HPLC method after five washes. Additional washes did not result in the removal of the bilayers associated with the microspheres (MS column). The large variance in the concentration in the

washing solution is due to the variation introduced by the removal of the supernatant solutions by pipetting.

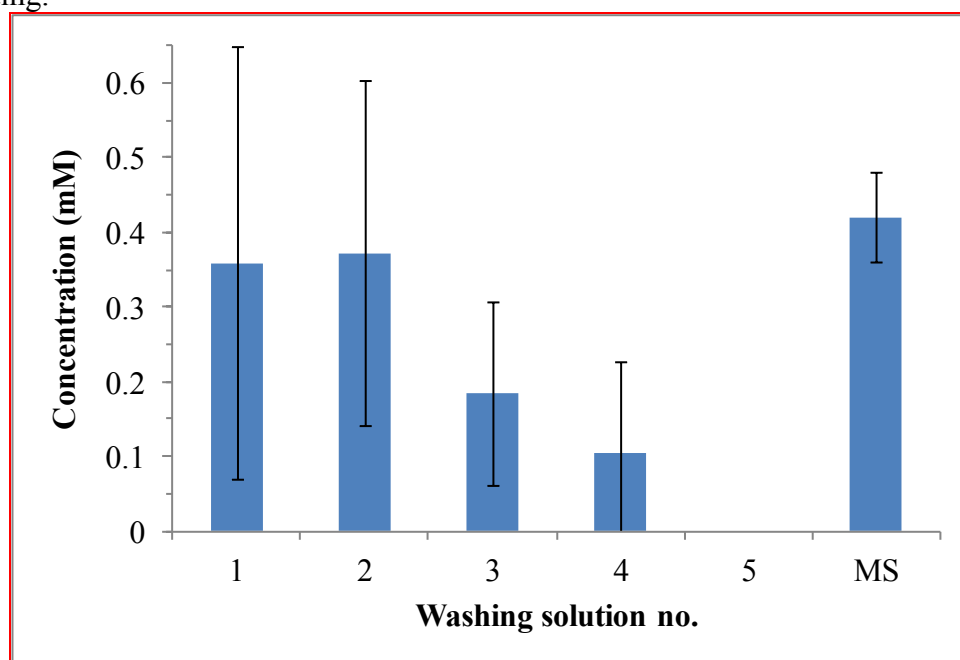


Figure SG. The association of biotinylated lipid anchored DA with the avidin-covered microspheres. Each washing step removed more of the unassociated DA from the samples until the 5th wash is performed. The large variation in the error bars is due to imperfect removal of the supernatant solution by pipetting. The MS column displays the amount of DA associated with the microspheres.

5.b: Association of ruthenium complex catalysis with the DA bilayers coating glass microspheres

The association of the lipophilic ruthenium complex with the anchored bilayers was also resistant to exchange of the hosting solution. After removal of the incubation solution, the microspheres were washed three times with buffer and the amount of ruthenium in each wash (washing solution) was measured using the intrinsic fluorescent signal of the metal complex as in Figure SH.

It was established that some lipophilic complex was strongly associated with the anchored bilayer. The determination of the associated ruthenium concentration therefore required the use of a detergent to be extracted. A similarly strong interaction between the ruthenium complex and DA bilayers has previously been demonstrated by Maurer *et al.*².

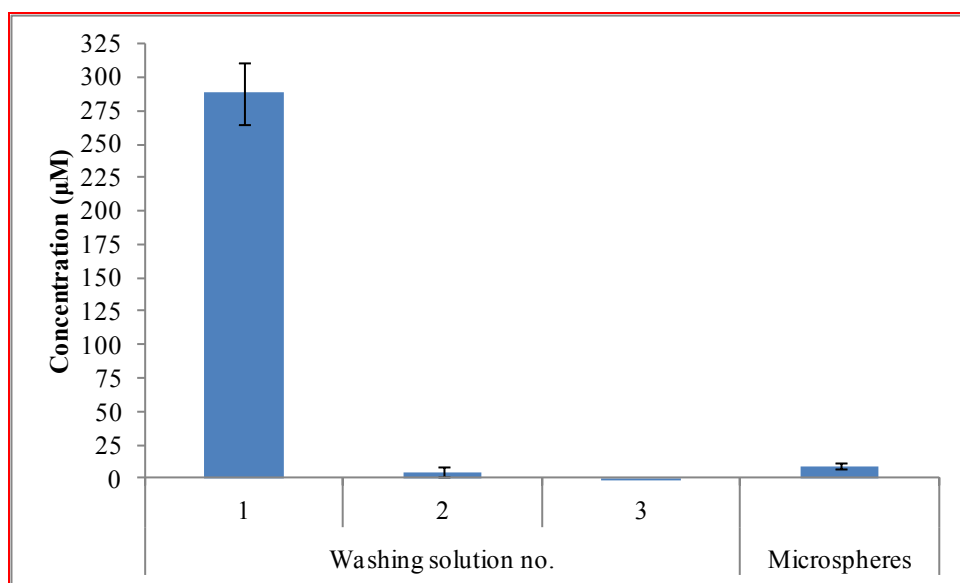


Figure SH. Association between the bilayer coated microspheres and the lipophilic ruthenium complex. The incubation solution contained 5 mM of the ruthenium complex while the washing solution contained no ruthenium complex. The amount of ruthenium complex associated with the microspheres is displayed for comparison. Note that the concentration of ruthenium in the 3rd washing solution was consistently below the detection limit.

In order to study the exchange of RuC10:0 between the ruthenium containing microspheres and a vesicle population further, the following experiment was performed. A microsphere population with anchored DA bilayers decorated with RuC10:0 was resuspended in 400 µL 20 mM DA at buffer. The ruthenium at an initial 10 µM concentration was exclusively located on the microspheres. After 24 hours of incubation on a shaker at 500 RPM, the vesicles were separated from the microspheres by centrifugation. To facilitate the quantification of the ruthenium complex by fluorimetry, the bilayers of both samples was disrupted using Triton X-100. A significant amount of the ruthenium complex 42 % (3.8 ± 1.6 µM) had been dispersed throughout the hosting solution, i.e., exchanged with the vesicles. The anchored bilayers had retained 58 %, (5.7 ± 1.1 µM) of the ruthenium complex. Thus, we cannot completely exclude this mechanism of exchange between generations as a means of catalytic property transmission. However, the concentration of vesicles was generally lower in the pL conversion experiments than in this experiment, which would reduce this type of exchange.

The anchoring of the ruthenium complex was sufficiently strong to withstand the exchange of the hosting solution by centrifugation, Figure SH. The associated ruthenium complex could be used to visualize the supported bilayers using fluorescence, Figure SI.

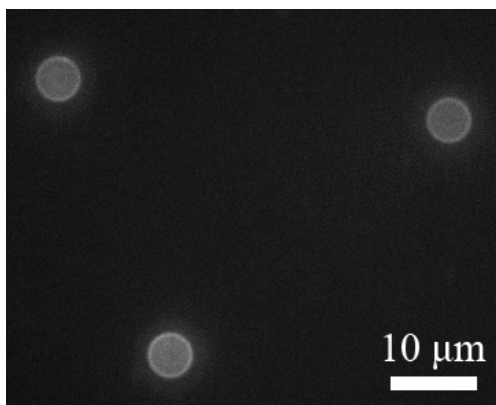


Figure SI. Micrograph of microspheres with anchored bilayers of decanoic acid, stained with a lipophilic ruthenium complex see the chemical structure in Figure SA. The decanoic acid-covered microspheres were incubated with 0.5 mM of ruthenium complex, RuC10:0. After several exchanges of the hosting solution to remove any metal complex not associated with the microspheres anchored bilayers. The density of ruthenium complex allowed for the visualization of the systems using the intrinsic fluorescence of the ruthenium. Bright edges on the surface of the microspheres indicate the presence of a multilamellar bilayer coating.

5.c: Association of pL with the DA vesicles and DA bilayers coating glass microspheres

The pL, which was supplied by slow injection of an ethanol solution into a vesicle suspension, associated strongly with DA vesicles, as demonstrated by the following FRET experiment.

The concentration of associated pL was determined using a standard curve and the relative donor fluorescence intensity, if the acceptor fluorescence intensity is normalized to a value of 100 for all samples. As the donor and acceptor molecules are separated (during growth) donor fluorescence intensity increases, giving a directly proportional relationship between the size and fluorescence. The resulting curve is not linear over the concentrations injected. Two standard curves were prepared one from 0-10 mM and one from 5-25 mM. The average values from the two standard curves were used as a final analysis, Figure SJ.

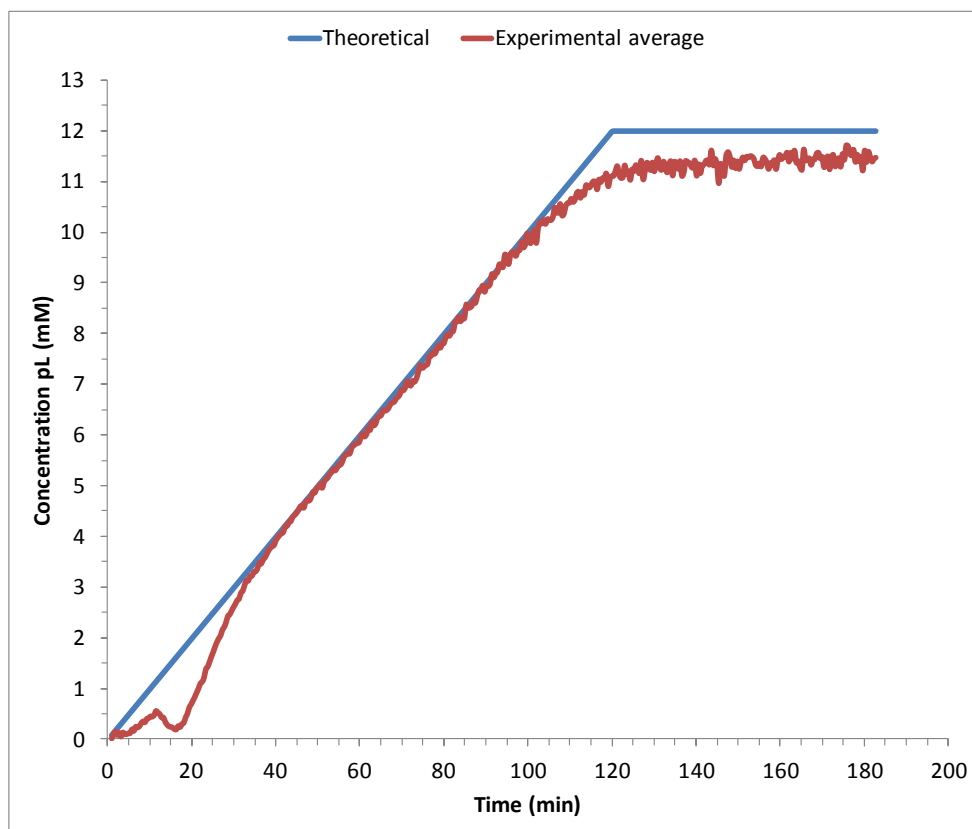


Figure SJ. Interaction between the pL and DA vesicles. The graph shows the association of the injected the pL during a slow-injection of 12 mM pL into 25 mM DA vesicles containing FRET probes. The graph also includes a theoretical curve for the instantaneous and quantitative interaction between the bilayers of the vesicles and the pL.

The insertion of injected pL into DA bilayers occurred rapidly and in an almost quantitative manner. The initial part of the curve likely followed the theoretical curve, however the sensitivity of this technique at low concentrations amplified any error in the measurement. Such sensitivity wasn't observed at higher concentrations. When the low concentration standard curve was used alone, the results in first 10 minutes matched the theoretical values almost perfectly.

The strong interaction between the pL and the DA bilayer was also monitored by fluorescence microscopy, Figure SK. The micrographs show a change in vesicle morphology as the concentration of the pL was increased during the slow-injection experiment. Note that the selected micrographs are shown to illustrate the striking differences between vesicles with/without pL.

The anchored bilayers display the same tendency to interact with the pL. However, the low concentration of fatty acid in the microsphere based system, when compared to DA vesicles, prevented the accommodation of comparable pL amounts. Slowly injecting concentrated pL solutions to a dispersion of microspheres in the absence of photocatalysis resulted in the aggregation of microspheres and the formation of oil droplets above a certain concentration ratio threshold, Figure SL.

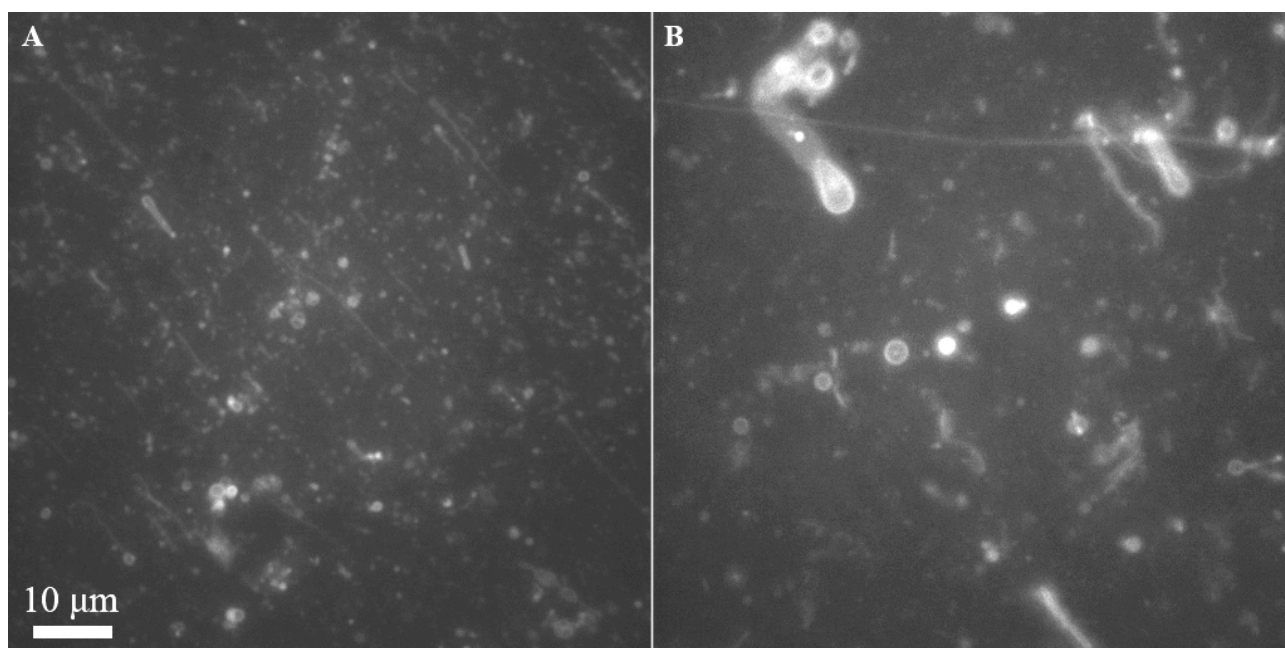


Figure SK. Interaction between DA vesicles and pL. Slow injection of pL to 30 mM of DA: (A) 0 mM and (B) 3.75 mM. The scale bar applies to both micrographs, the samples were stained with 0.1 mM Nile red.

During the slow-injection of pL the ratio between the pL and the fatty acid associated with the microspheres changed, Table SA.

Table SA. Ratio between pL and DA during the slow-injection of a 500 mM pL ethanol solution.

pL injected (mM)	pL/DA ratio
0	0.00
0.75	1.78
1.5	3.56
2.25	5.34
3	7.13
3.75	8.91
4.5	10.69
5	11.88

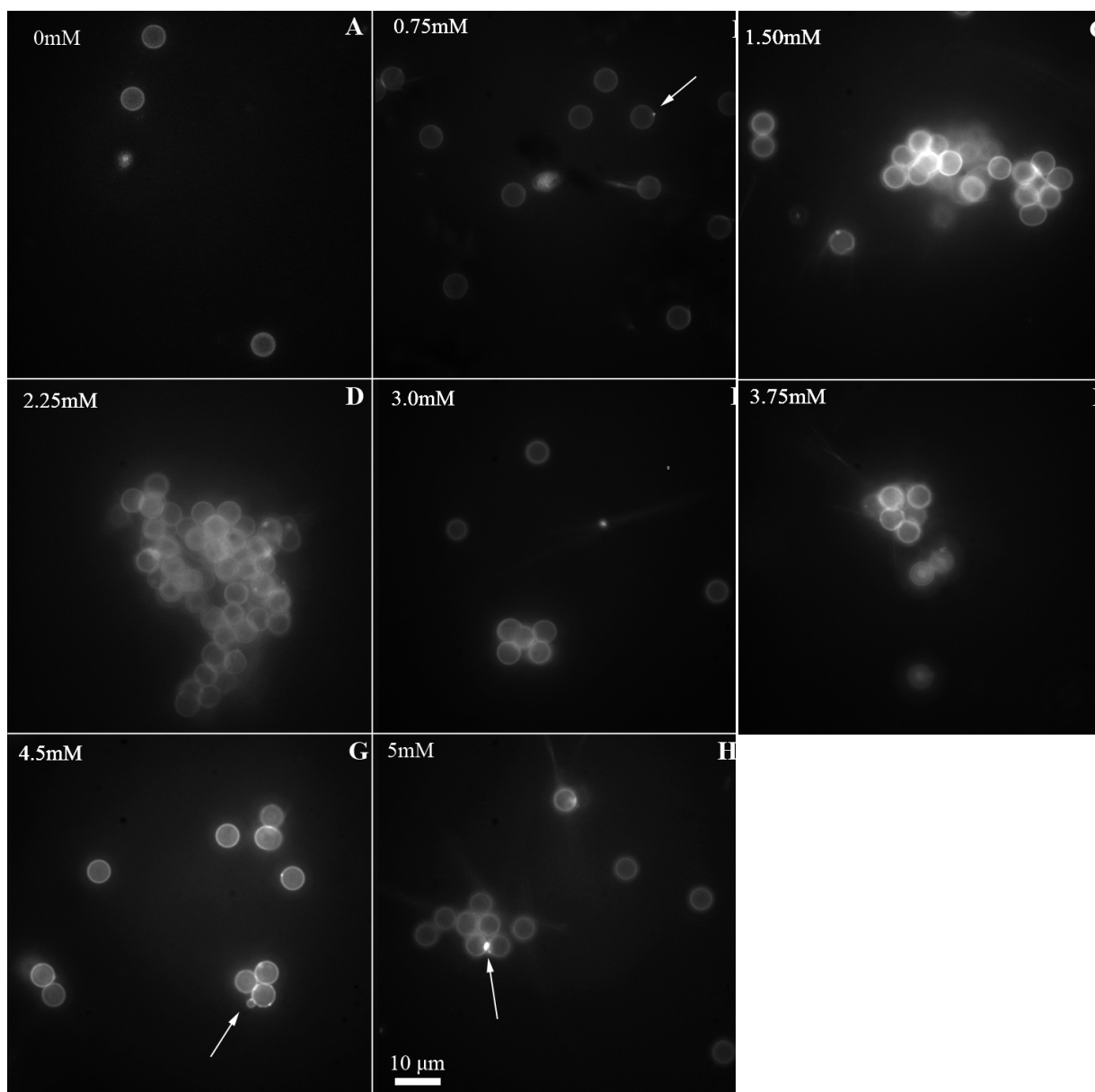


Figure S1. Slow injection of pL to microspheres in the absence of photoconversion. The morphological changes to the anchored bilayer during injection of up to 5 mM precursor are visible. The concentration of pL in the system is displayed in the top left corner in each micrograph. The arrows points to structures discussed in the text. The samples were stained with Nile red, the scale bar applies to all frames.

Based on the micrographs taken during the injection procedure, the maximum amount of pL accommodated by the microsphere system without morphological changes was around 0.75 mM, corresponding to 1.78 pL pL molecules per DA molecule. By contrast, in DA vesicle samples (larger DA concentrations), the final molar ratio of pL to DA corresponded to 0.48 pL molecules per DA molecule. Previous results² indicate that larger pL concentration destabilized the vesicles in a similar manner (i.e., aggregation of vesicles and the formation of oil inclusions in vesicle systems). The apparent enhanced tolerance to pL in the microsphere system could either be due to the real increase in tolerance from anchoring of the lipid bilayer or the exclusion of some precursor from the supports as independent oil droplets in the solution. However, none of the micrographs showed a significant amount of oil droplets, a fact that does not completely rule out their presence in the samples.

5.d: Photochemical conversion of pL on the microsphere protocells.

Once associated with the microspheres, the metabolic system was capable of photochemically converting the pL into DA. The reaction mixture contained 16.2 mM pL and 48 mM ascorbic acid. After a 28 h illumination period, the reaction was analysed by HPLC. The initial composition was compared to the illuminated sample and a non-illuminated sample, the latter was stored in the dark for the duration of the illumination of the reaction vessel, Figure SM. Clearly, the presence of 5 μm microspheres and the localization of the photocatalysis on their surface did not preclude an efficient pL conversion.

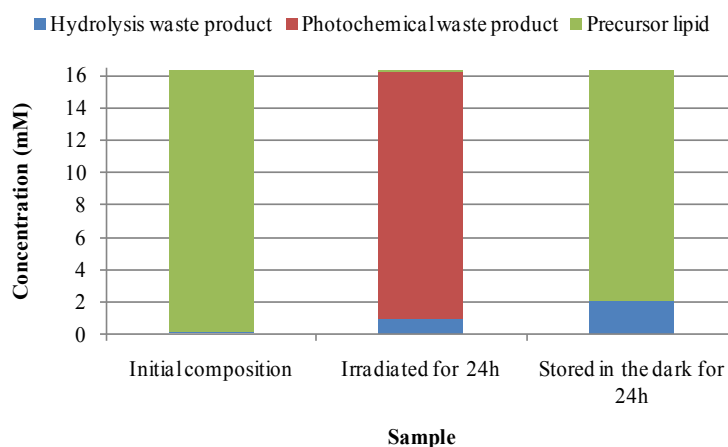


Figure SM. Production of amphiphile from the pL. HPLC analysis of a microsphere population with 16.2 mM pL, illuminated for 28 h. The graphs show the initial sample composition, the irradiated sample, and a control sample kept in the dark. The initial system contains 0.13 mM of hydrolysis product, either the remnant from the synthesis or the product of the solid compound hydrolysis during storage.

The bulk addition of pL resulted in extensive pL oil droplet formation. In order to minimize the amount of oil droplets during the irradiation of the microsphere systems, a slow injection procedure was used to supply them with a concentrated pL solution.

During the photochemical reaction, it was important to monitor the location of the photocatalyst, as the envisioned reaction would have to remain on the surface of the bilayer anchored to the microspheres. To determine the location of the photochemical complex, the fluorescent signal of the ruthenium complex was used to record micrographs of the solution (Figure SN). The micrographs show that ruthenium complexes were predominantly associated with the bilayers anchored to the microspheres up to 6 hours. The 24 h sample contained noticeable oil droplets containing ruthenium, although it is difficult to say whether these are a second generation aggregate or nascent oil droplets that had acquired dissociated ruthenium complexes.

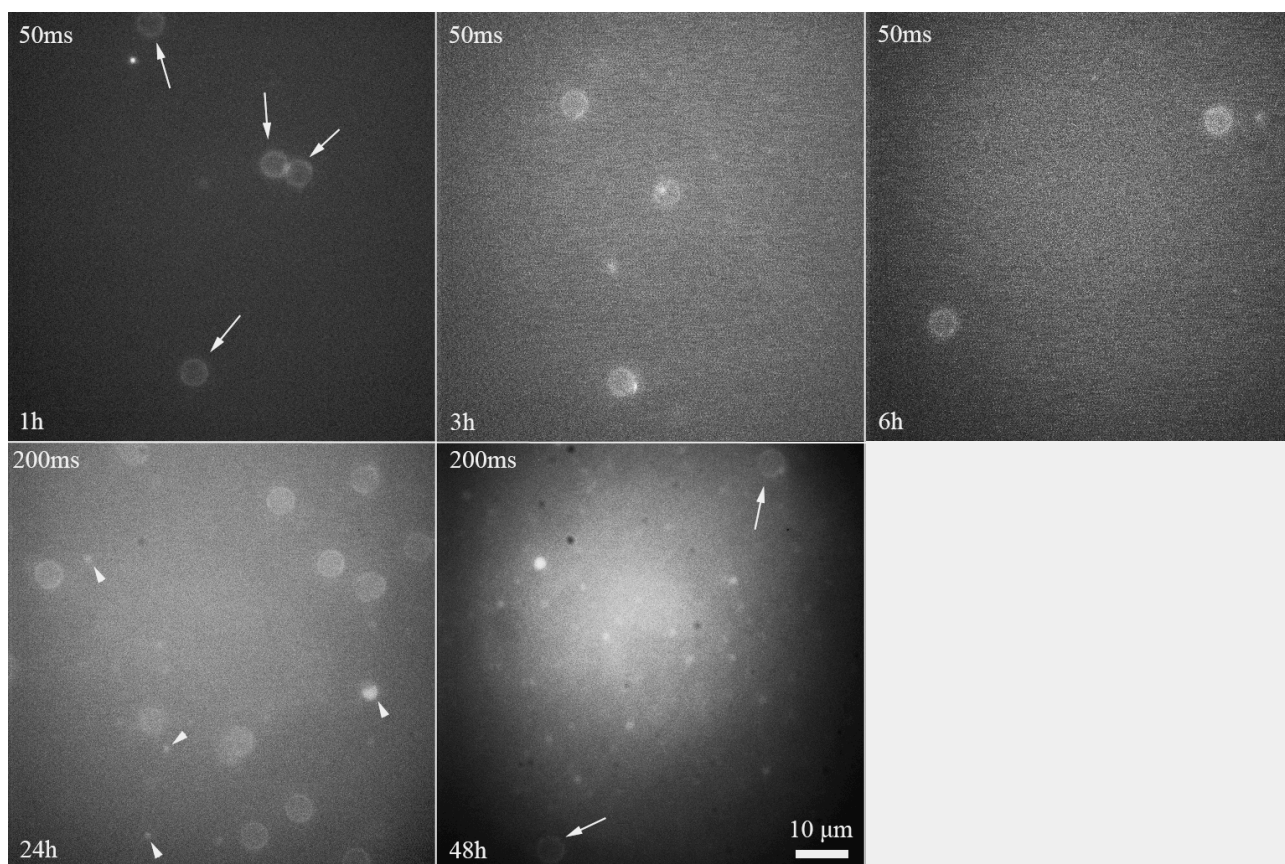


Figure SN. Micrographs displaying the location of the ruthenium complex at various time points during the slow-injection of the pL into the reaction chamber containing the microspheres with anchored bilayers decorated with the metabolic ruthenium complex. In micrographs arrows point to microspheres; arrowheads point to oil droplets. The intrinsic fluorescence of the ruthenium was used to monitor the reaction. Note that longer exposure times were necessary for the 24 and 48 hour samples because of a quenching of the ruthenium fluorescent signal as the illumination reaction progressed.

The samples were also stained with Nile red to better visualize the structures formed (Figure SO). A comparison of the Nile red and photocatalyst stained micrographs shows only a negligible amount of the ruthenium complex was dissociated from the bilayers anchored to the microspheres until 24 hours into the injection, a time at which the first vesicles could be observed in the sample. Thus, the increase of the fluorescence signal from photocatalyst was not associated with the microspheres coincided with the vesicle formation. The non-associated ruthenium was therefore likely located on newly formed vesicles. In general, the ruthenium complex was primarily located in DA bilayers, regardless of their anchoring on microspheres and no fluorescent, free-floating oil droplets were observed using the ruthenium signal.

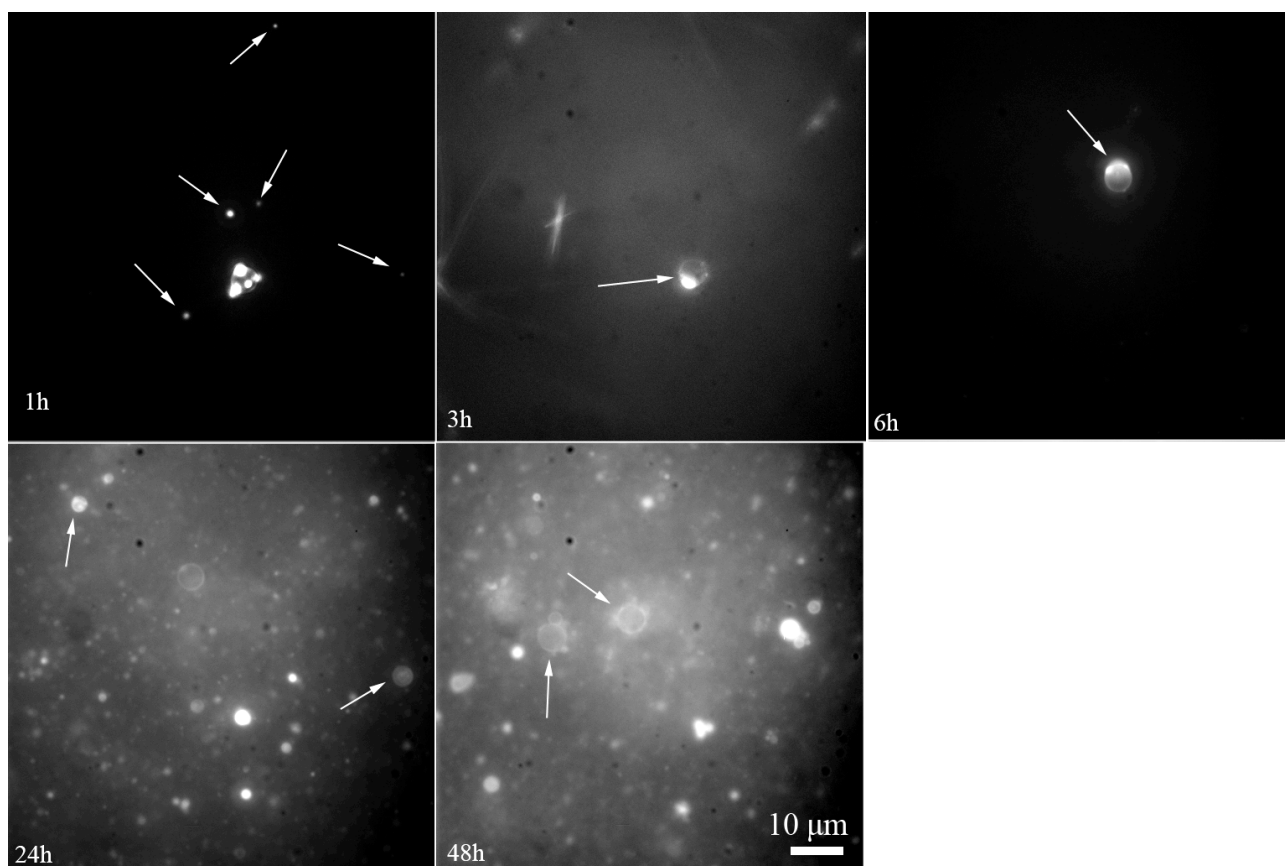


Figure SO. Micrographs of the photochemical reaction at different time points during the slow injection of the pL. Oil droplets associated with the microspheres are highlighted in the 1 h micrograph. The arrows points to oil inclusions in the microsphere bilayer at 3 h, 6 h and 48 h. In the 24 h micrographs the arrows highlight vesicles in the reaction solution. The micrographs were stained with Nile red, the scale bar applies to all micrographs.

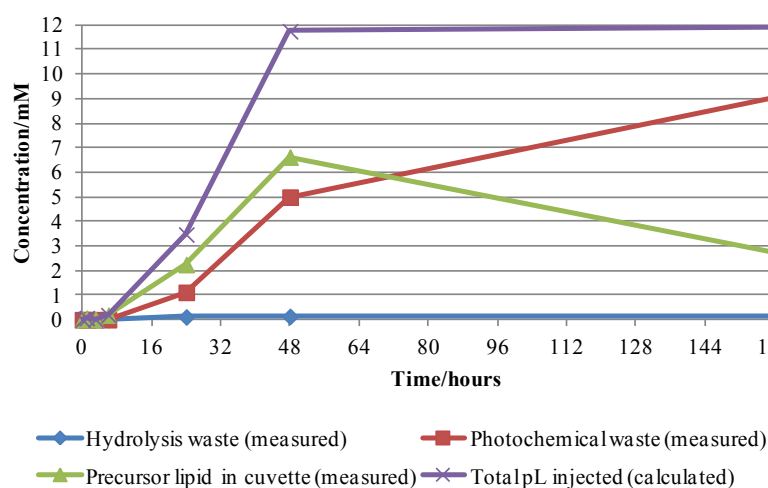


Figure SP. The change in the composition of the illuminated sample during photochemical reaction, the composition was determined by HPLC analysis.

The reaction was monitored by HPLC during the illumination (Figure SP). The onset of vesicle formation occurred between 6 and 24 hours, which corresponded with the timeframe where the amount of photochemical waste product increased significantly. After the formation

of vesicles, the photochemical conversion of the pL further increased in rate, which indicated that the conversion of precursor was influenced by the formation of new reaction surface and not simply depended on the availability of pL. This observation could be explained by the fact that the formation of vesicles would have exposed more bilayer surface, i.e., a higher concentration of catalyst, to the ascorbic acid, as the concentric bilayers on the microspheres were shed away.

During the photochemical conversion of 12 mM pL, a single ruthenium complex molecule would have performed on average 1344 photochemical turnovers.

5.e: 8-oxoguanine based reaction

The 8-oxoguanine mediated photochemical reaction as described by DeClue *et al.*¹, was compatible with the microsphere-anchored bilayer. The oxygen sensitivity of this reaction prevented the use of slow-injection to deliver pL into the reaction chamber. Instead, the pL was added in bulk before the sample was illuminated. The result of 121 hours of the illumination of a microsphere population with 6 mM pL is presented below (Figure SQ).

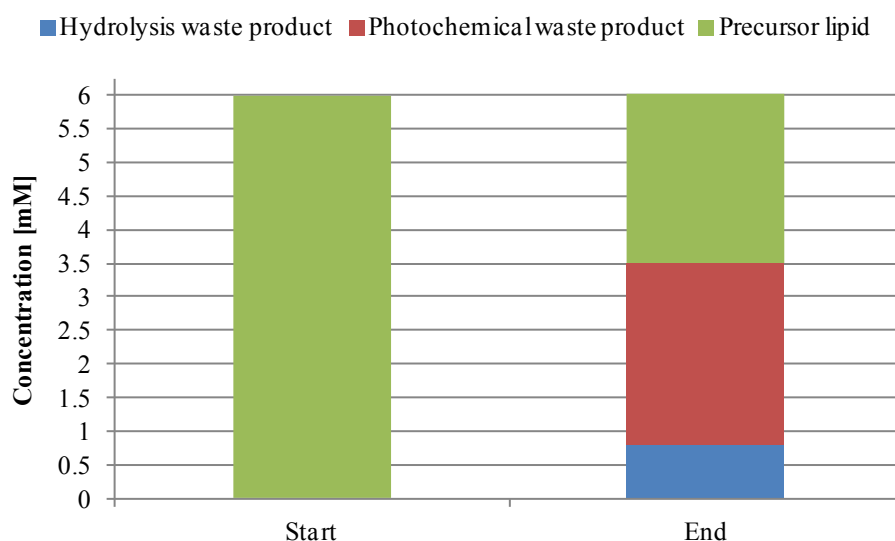


Figure SQ. HPLC analysis of pL conversion using the 8- oxoG-RuC10:0 complex. The system was illuminated for 121 hours before HPLC analysis.

Previous experiments showed that the 8-oxoguanine reaction was significantly slower than the ascorbic acid-based reaction. The lower reaction rate did not change the primary conversion route (photochemical cleavage of the pL ester bond). When compared with the experiments by Maurer *et al.*² and DeClue *et al.*¹, the pL conversion rate of the microsphere system was significantly lower due to the difference in the pL to Ru ratio. In this paper, the ratio was approximately 600:1 compared that of 5:1 and 50:1² and the 15:1¹. Such a difference can be expected to result in lower reaction rates. However, even with this slow rate of reaction, the rate of hydrolysis was still a secondary pathway for DA formation.

6. References

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