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Preparation of Giant Vesicles Exhibiting Visible-Light-Induced Morphological Changes

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Abstract:	We describe the preparation of giant vesicles that incorporate a photoresponsive ruthenium complex having two alkyl chains. The vesicles exhibit morphological changes when exposed to visible light. The ruthenium complex proximal-[Ru(L1)(L2)OH2](NO3)2, proximal-2 (L1 is 4'-decyloxy-terpyridine, L2 is 2-(2'-(6'-decyloxy)-pyridyl)quinoline) was prepared by thermal reaction of Ru(L1)Cl3 and L2, followed by removal of a chloride ligand. In an aqueous solution and vesicle dispersion, proximal-2 was reversibly photoisomerized between proximal and distal isomers. Giant vesicles containing proximal-2 were prepared by hydration of phospholipid films containing proximal-2 in the dark at 80 °C. Giant vesicles were frequently formed from DOPC/proximal-2 vesicles rather than from DPPC/proximal-2 vesicles (DOPC is 1,2-dioleoyl-sn-glycero-3-phosphocholine, DPPC is 1,2-dipalmitoyl -sn-glycero-3-phosphocholine). The ratio of proximal-2 and DOPC in the vesicle preparation was varied from 5:100 to 20:100. The light-induced morphological changes were observed for proximal-2/DOPC in the presence of Na2SO4. However, they were highly suppressed in the presence of NaOH. Morphological changes were observed under fluorescence microscopy using 635-nm (red) light. Rhodamine-DOPC [rhodamine-DOPC: 1,2-dioleoyl-sn-glycero-3-phos-phoethanolamine-N-(lissamine rhodamine B sulfonyl)] was used to fluorescently label the vesicles.
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TITLE: Preparation of Giant Vesicles Exhibiting Visible-Light-Induced Morphological Changes

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SHORT ABSTRACT:
The synthesis of ruthenium complex surfactants exhibiting photoisomerization in giant vesicles is described. The preparation and light irradiation of the giant vesicles are also described.

LONG ABSTRACT:
We describe the preparation of giant vesicles that incorporate a photoresponsive ruthenium complex having two alkyl chains. The vesicles exhibited morphological changes when exposed to visible light. The ruthenium complex *proximal*-[Ru(**L1**)(**L2**)OH_2]$(NO_3)_2$, *proximal*-**2** (**L1** is 4'-decyloxy-2,2';6',2"-terpyridine, **L2** is 2-(2'-(6'-decyloxy)-pyridyl)quinoline) was prepared by a thermal reaction of Ru(**L1**)Cl_3 and **L2**, followed by removal of a chloride ligand. In an aqueous solution and vesicle dispersions, *proximal*-**2** was reversibly photoisomerized to the distal isomer. Giant vesicles containing *proximal*-**2** were prepared by hydration of phospholipid films containing *proximal*-**2** in the dark at 80 °C. Giant vesicles were frequently found in the dispersions prepared from DOPC/*proximal*-**2** rather than from DPPC/*proximal*-**2** (DOPC is 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, DPPC is 1,2-dipalmitoyl -*sn*-glycero-3-phosphocholine). The ratio of *proximal*-**2** and DOPC in the vesicle preparation was varied from 5:100 to 20:100. The light-induced morphological changes were observed for *proximal*-**2**/DOPC in the presence of Na_2SO_4. However, they were highly suppressed in the presence of NaOH. Incubation of light-exposed vesicles at 45 °C in the dark induced reverse morphological changes. Morphological changes were observed under fluorescence microscopy using 635 nm (red) light. Rhodamine-DOPC [rhodamine-DOPC: 1,2-dioleoyl-sn-glycero-3-phos-phoethanolamine-N-(lissamine rhodamine B sulfonyl)] was used to fluorescently label the vesicles.

INTRODUCTION:
Controlling the morphologies and shapes of macro- and meso-scale molecular assemblies by external stimuli has attracted considerable attention.[1,2] In particular, the control of vesicle morphologies by remote stimuli such as light has potential applications for drug delivery.[3] In this context, organic photochromic molecules with hydrophobic and hydrophilic moieties have been widely incorporated into liposomes and polymer vesicles.[4-8] However, most of the assemblies require ultraviolet (UV) light to drive the morphological changes, and their applications are limited because UV light is strongly scattered in living tissues and induces DNA damage and cell death.

Alternatively, utilization of visible or near-infrared light in the phototherapeutic window (600–1000 nm) is more favorable because of abundant sunlight and its high transmission in tissues of living organisms. In this regard, ruthenium complexes with polypyridyl ligands are suitable visible-light-responsive surfactants. They exhibit a strong visible light absorption band ($\varepsilon \sim 10^4$ M^{-1} cm^{-1}) that induces ligand substitution[9,10] and photoisomerization.[11-16] Incorporation of the ruthenium complexes into vesicles will expand their applications because these complexes are also known as water oxidation catalysts[17-19] and bioactive molecules.[20,21] Recently, ruthenium complexes have been incorporated into vesicles.[22-24] However, controlling morphologies of vesicles *via* visible-light absorption has remained challenging.

We have previously reported irreversible and reversible photoisomerization of mononuclear ruthenium aqua complexes having asymmetric bidentate ligands.[25-28] Recently, we synthesized novel surfactants (*proximal*-**2**, see Figure 1) that exhibit visible-light photoisomerization equilibria with *distal*-**2** by introducing an alkyl chain on each tridentate and bidentate ligand of the ruthenium aqua complex. Giant vesicles incorporating *proximal*-**2** undergo morphological changes under the irradiation of visible light in the phototherapeutic window.[29] Herein, we describe the detailed syntheses of ruthenium complexes and the preparation of giant vesicles. The protocols will enable researchers to prepare, characterize, and utilize light-responsive giant vesicles.

[Place Figure 1 here]

PROTOCOL:

Note: Ru(tpy)Cl_3[30], **L1**[29], 2-(2'-(6'-chloro)-pyridyl)quinoline[29], *proximal*-**1**[29] were synthesized as previously described.

1. Synthesis of 2-(2'-(6'-decyloxy)-pyridyl)quinoline (L2)

1.1) Add 2-(2'-(6'-chloro)-pyridyl)quinoline (16.3 mg, 63 µmol), 1-decanol (0.1 mL), dimethyl sulfoxide (1 mL), KOH (0.12 g) to a 50 mL round bottom flask equipped with a stir bar.

1.2) Heat and stir the reaction mixture in an 80 °C oil bath for 4 h.

1.3) Transfer the reaction mixture to a separating funnel, and add chloroform (*ca.* 20 mL) and water (*ca.* 20 mL). Shake the funnel for 2–3 minutes and wait several hours for complete separation into two layers. Collect the bottom organic layer, add anhydrous magnesium sulfate to absorb the water in the chloroform, filter with folded filtration paper, and remove the solvent in a rotary evaporator at 40 °C to obtain the oily crude product.

1.4) Purify the product with silica gel chromatography (1.5 cm×20 cm) using a mixed solvent (AcOEt/hexane/$CHCl_3$, 1:5:5, v/v/v) as an eluent.[29] The product band emits blue light in the silica gel under UV light (254 nm).

1.5) Collect the fractions of the blue band eluted from the column to the glass vials, and remove the solvent in a rotary evaporator at 40 °C. Check the product purity with ^1H and ^{13}C NMR in $CDCl_3$ referenced with tetramethylsilane (TMS). The oily product contains a small amount of 1-decanol as impurity.[29]

2. Synthesis of *proximal*-2

2.1) Synthesis of [Ru(L1)Cl_3]

2.1.1) Add $RuCl_3 \cdot 3H_2O$ (60 mg, 0.23 mmol), **L1** (80 mg, 0.21 mmol), and ethanol (EtOH, 40 mL) to a 50 mL round bottom flask equipped with a stir bar.

2.1.2) Reflux and stir the reaction mixture in an oil bath for 4 h.

2.1.3) Collect the yellow-brown precipitate with vacuum filtration, and wash with water (*ca.* 5 mL).

2.1.4) Dry the product in vacuum for a yield of approximately 98 mg (80% yield).

2.2) Synthesis of [Ru(L1)(L2)Cl]Cl

2.2.1) Add [Ru(**L1**)Cl_3] (47.2 mg, 0.078 mmol), triethylamine (0.2 mL), EtOH (12mL), water

(4mL), LiCl (50 mg), and purified product of **L2** synthesized from 0.10 mmol of 2-(2'-(6'-chloro)-pyridyl)quinoline to a 50 mL round bottom flask equipped with a stir bar.

2.2.2) Reflux the reaction mixture in an oil bath for 4 h.

2.2.3) Filter the purple solution with diatomite (*ca.* 2 g) on the filter paper in a glass funnel to remove unreacted [Ru(**L1**)Cl_3].

2.2.4) Reduce the solvent to *ca.* 3 mL in a rotary evaporator at 45 °C, collect the purple precipitate by vacuum filtration, and wash with diethyl ether.

2.2.5) Purify the crude solid (44.2 mg) with size exclusion chromatography on a dextran gel, using methanol as eluent (column length: 20 cm).[29] Collect fractions of the second purple band eluted from the column to glass vials. Spot the eluted fractions on the thin layer chromatography plate (*ca.* 1 µL for each spot). Check the purity using a mixed eluent (MeOH/saturated aqueous solution of NaCl, 30:1, v/v). Repeat the purification process two or three times.

2.2.6) Remove the solvent on a rotary evaporator at 45 °C and dry in vacuum to obtain 30.1 mg of the product (39% yield). Check the purity with by ^{1}H and ^{13}C NMR in $CDCl_3$ referenced with TMS.[29]

2.3) Synthesis of *proximal*-[Ru(L1)(L2)OH_2]$(NO_3)_2$ (*proximal*-2)

2.3.1) Add *proximal*-[Ru(**L1**)(**L2**)Cl]Cl (16.3 mg, 0.017 mmol), water (3 mL), acetone (10 mL), and an aqueous solution of 0.1 M $AgNO_3$ (0.60 mL, 0.060 mmol) to a 50 mL round bottom flask equipped with a stir bar. Cover the flask with aluminum foil

2.3.2) Reflux the reaction mixture in an oil bath for 2 h in the dark.

2.3.3) Filter the purple solution with diatomite (*ca.* 2 g) on the filter paper in a glass funnel.

2.3.4) Reduce the solvent to *ca.* 3 mL in a rotary evaporator at 45 °C, collect the purple solid, and wash it with water.

2.3.5) Dry in vacuum to obtain 12.6 mg of the product (75% yield). Check the purity with ^{1}H and ^{13}C NMR in the mix solvent of *d*-acetone and D_2O (1:1, v/v) referenced with TMS.[29]

3. Standard conditions for preparation of vesicles

3.1) To prepare 0.5 mM stock solution A, dissolve 4.0 mg of *proximal*-**2** in 8.0 mL of chloroform. Store the stock solution in the dark and refrigerate.

3.2) To prepare 1.0 mM stock solution B, dissolve 15.7 mg of DOPC in 20.0 mL of chloroform.

3.3) To prepare 0.1 mM stock solution C, dilute 1 mg/mL solution of rhodamine-DOPC with 6.6 mL of chloroform.

3.4) Mix 40 µL of stock solution A and 100 µL of stock solution B in an amber glass vial. For

the fluorescence microscopy experiments, add 100 µL of stock solution C.

3.5) Seal the vial with a rubber septum equipped with a nitrogen inlet and outlet, and dry the solution under nitrogen flow overnight.

3.6) Remove the septum and heat the vial in an 80 °C oven for 30 min.

3.7) Add 0.1 mL of pure water to the vial. Seal and incubate the vial at 80 °C overnight. The lipid film is gradually hydrated to yield a reddish-purple vesicle dispersion that settles to the bottom of the vial.

3.8) Store the vial in a refrigerator in the dark. The vesicle dispersion should be used within a week.

4. Preparation of Plates

4.1) Rinse glass plates with ethanol and acetone, sonicate in ethanol for 5 min, and dry at 50 °C for 20–30 min.

4.2) Cut a silicon film (thickness = 0.2 mm) into a 20 mm×20 mm square with a knife.

4.3) Make a 5 mm hole with a hole punch at the center of the film, and remove plastic covers.

4.4) Wet one side of the silicon film with diluted detergent (0.3 %) and then wipe it with cleaning tissue.

4.5) Attach the edge of the film to a glass plate, and slowly lay the film in order to extrude the bubbles.

4.6) Slowly shake the amber vial containing the vesicles, and with a micropipette transfer a small drop (diameter ~1 mm) to the center of the hole on the glass plate.

4.7) Place a cover glass (18 mm × 18 mm) on the vesicle dispersion.

5. Morphological changes of giant vesicles under visible light irradiation

5.1) Perform experiments in the dark at a constant temperature of 25 °C.

5.2) Put the glass plate with sample droplets under a digital microscope (700X), and acquire images.

5.3) Expose the sample plate with emission from a halogen lamp (distance from the plate: 2.5 cm) at a constant intensity of 120 $mWcm^{-2}$.

6. Morphological changes of giant vesicles under red light irradiation

6.1) Perform experiments in the dark at a constant temperature of 25 °C.

6.2) Put the glass plate with sample droplets containing DOPC, *proximal*-**2**, and rhodamine-

DOPC under a confocal microscope.

6.3) Acquire images with a confocal microscope. Transmit excitation light (559 nm) and emission light (575 nm) through the same objective.

6.4) Turn on the LED laser (635 nm), and adjust its intensity to 20 mW.

REPRESENTATIVE RESULTS:

We obtained high-purity *proximal*-**2** to form spherical and giant multilamellar vesicles (*proximal*-**2**/DOPC, *proximal*-**2**: DOPC=20:100) 15-μm average diameters (see Table 1).[29] Several layers were found inside the vesicles (Figures 2A and 2C). The inner spheres of the vesicles in Figures 2B, and 2D were darker than the outer spheres because of the concentric lipid layers. The vesicles containing *proximal*-**2** displayed various morphological changes under the irradiation of visible light ($\lambda > 380$ nm). The vesicle diameter in Figure 2A both increased and then decreased, while that in Figures 2B was distorted and had budding. The morphological changes were not usually observed for vesicles prepared from DOPC alone. Most of the morphological changes depended on the amount of *proximal*-**2** (Table 1). Changes were also observed for *proximal*-**2**/DPPC vesicles (DPPC=1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine). In contrast, *proximal*-**1**/DOPC vesicles did not show visible-light-induced morphological changes.

Figure 4 shows vesicles prepared in the presence of Na_2SO_4 (Figure 4A) and NaOH (Figure 4C). The photoinduced morphological changes were frequently observed for the vesicles containing Na_2SO_4 (Figure 4B) while those were not observed for the vesicles containing NaOH (Figure 4D). Figure 5 shows photo- and thermal-induced morphological changes of vesicles of *proximal*-**2**/DOPC. The budded vesicles under light irradiation recovered the original spherical vesicle after incubation in the dark.

The morphological changes in *proximal*-**2**/DOPC/rhodamine-DOPC vesicles under red light (635 nm) irradiation are depicted in Figure 6. We observed budding of granule vesicles from the vesicle edges, which is similar to that observed when exposed to visible light ($\lambda > 380$ nm).

Figure Legends:

Table 1: Dependence of morphological changes on vesicle preparation parameters. The percentages of vesicles showing morphological changes were calculated from vesicles (>10 mm) under visible light irradiation ($\lambda > 380$ nm, 120 $mWcm^{-2}$) for 30 min.

Figure 1: Ruthenium complex surfactants. Reversible photoisomerization equilibrium between *proximal*- and *distal*- type complex of **1** (top) and **2** (bottom).

Figure 2: Microscope images of vesicles under irradiation with a 100 W halogen lamp (λ>380 nm, 120 $mWcm^{-2}$). A) and B): *proximal*-**2**/DOPC (DOPC: 100 nmol, *proximal*-**2**: 20 nmol (20 mol%), water 0.1 mL). C) Vesicles prepared from DOPC alone (DOPC: 100 nmol, water 0.1 mL). D): *proximal*-**2**/DPPC (DPPC: 100 nmol, *proximal*-**2**: 20 nmol (20 mol%), water 0.1 mL). Parts reproduced from ref[29] with permission of John Wiley and Sons, Inc.

Figure 3: Microscope images of vesicles before light exposure. A), *proximal*-**2**/DPPC (DPPC:

100 nmol, *proximal*-**2**: 20 nmol (20 mol%), water 0.1 mL). B), *proximal*-**2**/DOPC (DOPC: 100 nmol, *proximal*-**2**: 20 nmol (20 mol%), water 0.1 mL).

Figure 4: Microscope images of vesicles under the irradiation with a 100 W halogen lamp (λ>380 nm, 120 mWcm^{-2}) in the presence of ionic compounds. A) and B), *proximal*-**2**/DOPC (DOPC: 100 nmol, *proximal*-**2**: 20 nmol (20 mol%), NaOH: 1000 nmol, water 0.1 mL). C) and D), *proximal*-**2**/DOPC (DOPC: 100 nmol, *proximal*-**2**: 20 nmol (20 mol%), Na_2SO_4: 500 nmol, water 0.1 mL). Left panels: before light irradiation and right panels: after light irradiation for 27 min with a 100 W halogen lamp.

Figure 5: Photo- and thermal-induced morphological changes. *proximal*-**2**/DOPC (DOPC: 100 nmol, *proximal*-**2**: 20 nmol (20 mol%), water 0.1 mL). The vesicle dispersions were irradiated under visible light at 25 °C (top) and then incubated in the dark at 45 °C (bottom). Scale bar: 50 μm.

Figure 6: Morphological changes of giant vesicles exposed to 635-nm light. Confocal fluorescence microscope (A and C), and digital microscope (B and D) images of giant vesicles containing DOPC (100 nmol), *proximal*-**2** (20 nmol), and rhodamine-DOPC (10 nmol) under irradiation with a diode laser (635 nm, 20 mW). The fluorescence microscopy was acquired with excitation at 559 nm excitation. Scale bar: 30 μm. Reproduced from ref[29] with permission of John Wiley and Sons, Inc.

DISCUSSION:

The ruthenium chloro complex *proximal*-[Ru(**L1**)(**L2**)Cl]$^+$ was prepared by thermal synthesis of Ru(**L1**)Cl_3 and a bidentate ligand **L2** in the presence of triethylamine. The proximal isomer was the major product and a distal isomer and Ru(**L1**)$_2^{2+}$ was a minor impurity. The crude product was purified with size-exclusion chromatography using methanol as an eluent. Coordinating solvents, such as water and acetonitrile, should not be used. Slow dropping of the eluent (3–4 drops per minute) is required to separate the product from impurities. The product purification can be performed under room light because *proximal*-[Ru(**L1**)(**L2**)Cl]$^+$ does not photoisomerize in methanol. The aquation of *proximal*-[Ru(**L1**)(**L2**)Cl]$^+$ to *proximal*-[Ru(**L1**)(**L2**)OH_2]$^{2+}$ (*proximal*-**2**) should be performed in the dark to prevent photoisomerization of the product.

Giant vesicles were prepared by simple hydration of lipid films containing the phospholipids and *proximal*-**2**. The DOPC and *proximal*-**2** vesicles were spherical and multilamellar, while those obtained from DPPC and *proximal*-**2** were slightly distorted as depicted in Figure 2. More giant vesicles were formed from *proximal*-**2**/DOPC than from *proximal*-**2**/DPPC, as depicted in Figure 3. The hydration temperature of the films should be more than 50 °C; giant vesicles were not formed after room-temperature hydration. The hydration time was varied over 5–24 hours with no significant differences in vesicle morphologies. After film hydration, the vesicle-containing samples were stored in the dark at 4 °C and used within a week. The vesicles can be prepared in the presence of ionic compounds such as Na_2SO_4 and NaOH, as depicted in Table 1. As shown in Figures 4C and 4D, the morphological changes were highly suppressed in the presence of NaOH (Figure 4C and 4D). The results arise from the formation of ruthenium-hydroxo complex (Ru-OH), which has been reported as inactive for photoisomerization.[28]

We previously reported that the mixture of *proximal*- and *distal*-**2** in the photostationary state displayed thermal back isomerization to the proximal isomer in an aqueous solution at 45 °C. In the vesicle dispersions, vesicles were irradiated under the visible light at 25 °C, and then incubated in the dark at 45 °C as depicted in Figure 5. The vesicles displayed budding from the edge under the light irradiation due to the photoisomerization to *distal*-**2**. The budded vesicles were recovered to the original spherical vesicles after incubating the vesicle in the dark at 45 °C for 30 min. The back morphological change may arise from the thermal back isomerization of *distal*-**2** to *proximal*-**2**.

Fluorescence microscopy was used to examine the fluorescent surfactants rhodamine-DOPC and fluorescein-DOPC [fluorescein-DOPC is 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(carboxyfluorescein) ammonium salt]. No fluorescence was observed for *proximal*-**2**/DOPC/fluorescein-DOPC vesicles because of the overlapping emission band of the fluorescein dye with a metal-to-ligand charge-transfer absorption band of *proximal*-**2**. In contrast, rhodamine-DOPC can be used in fluorescence experiments because of its red emission (575 nm). The percentage of the rhodamine-DOPC (20 mol %DOPC) was higher than previous studies[6] because the absorption of rhodamine-DOPC overlapped with that of *proximal*-**2**.

Common troubleshooting tips for the protocols are: (a) clean the amber vials before the preparation; (b) gently evaporate the chloroform from the lipids and the ruthenium complex under nitrogen gas flow; and (c) protect the samples from light before the measurements.

Generally, giant vesicles have been prepared from simple hydration, electroformation, or centrifugation methods.[31] Electroformation methods have been widely used for the preparation of giant unilamellar vesicles. However, we did not adopt the method in order to avoid redox reactions of the ruthenium complex under the electric field. In this protocol, we prepared giant multilamellar vesicles by hydration of lipid films with distilled water or aqueous solutions containing 10^{-4} M ionic compounds. It should be noted that it is difficult to prepare giant vesicles of *proximal*-**2**/DOPC in a highly concentrated aqueous solution of ionic compounds ($> 10^{-2}$ M). The red light responsive vesicles of *proximal*-**2**/DOPC are contrastive to the UV-light responsive vesicles reported so far.[6-8] We are now trying to prepare the giant vesicles containing the ruthenium complex under physiological conditions.

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The authors have nothing to disclose.

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Figure 1

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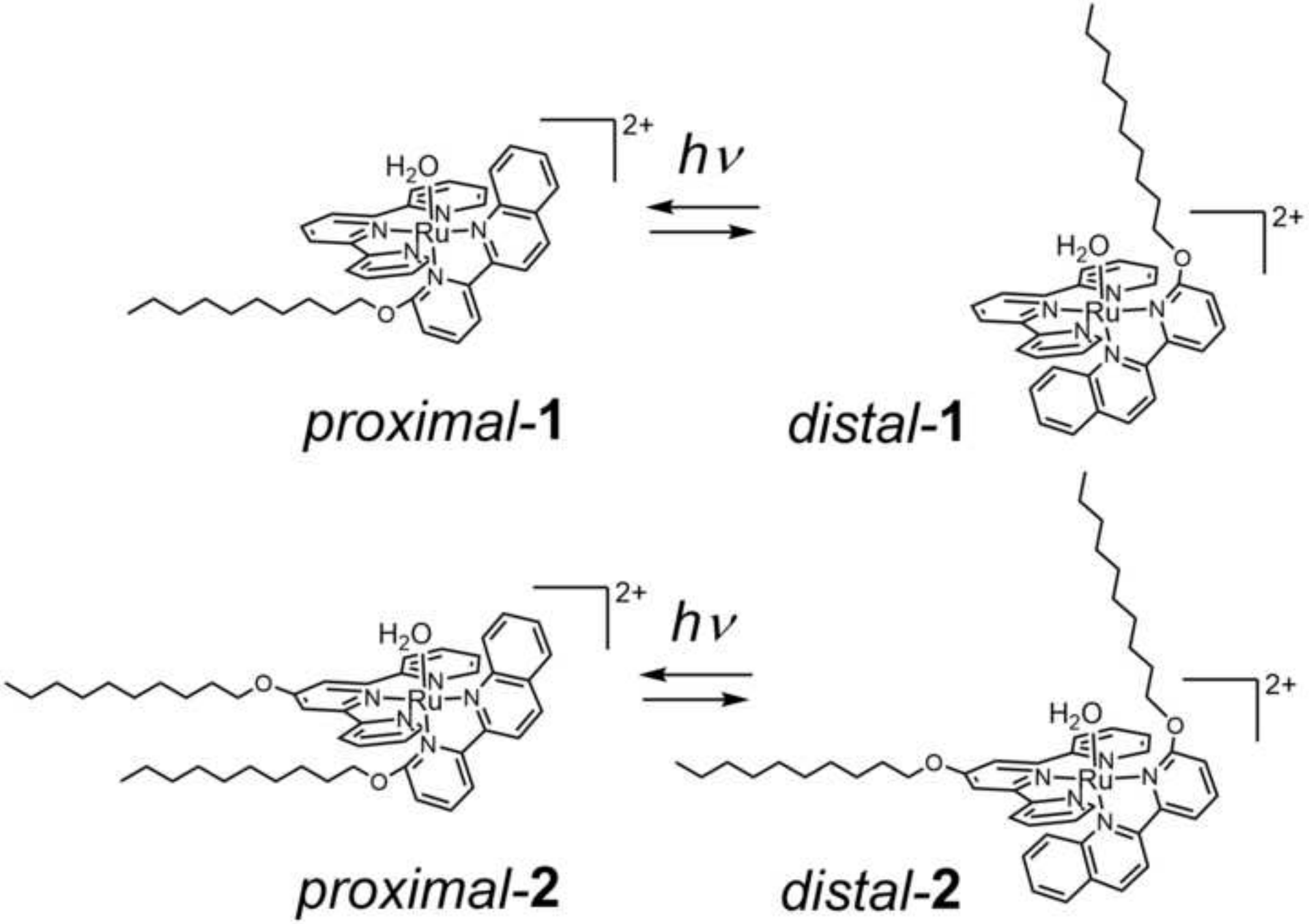

Figure 2

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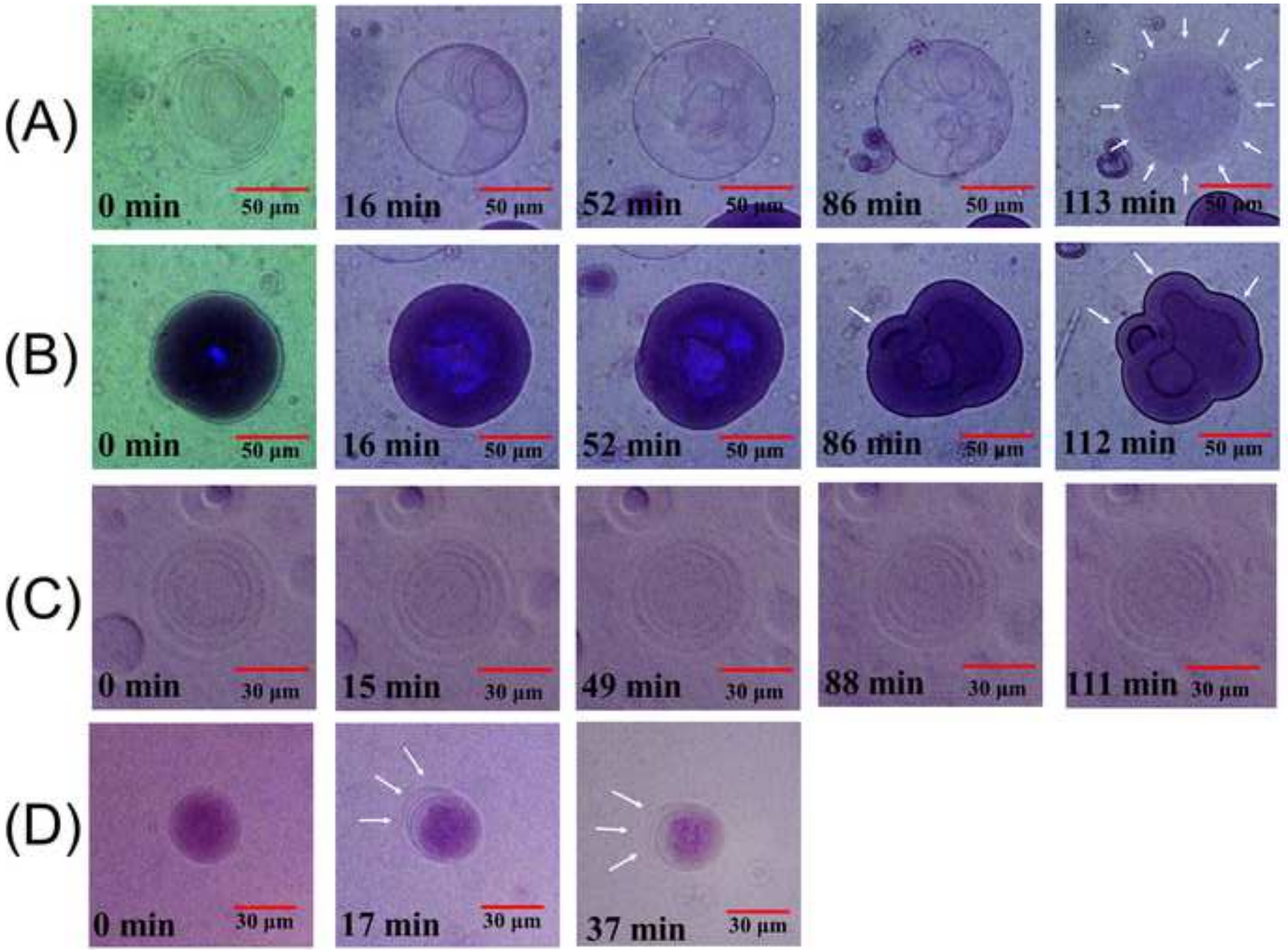

Figure 3

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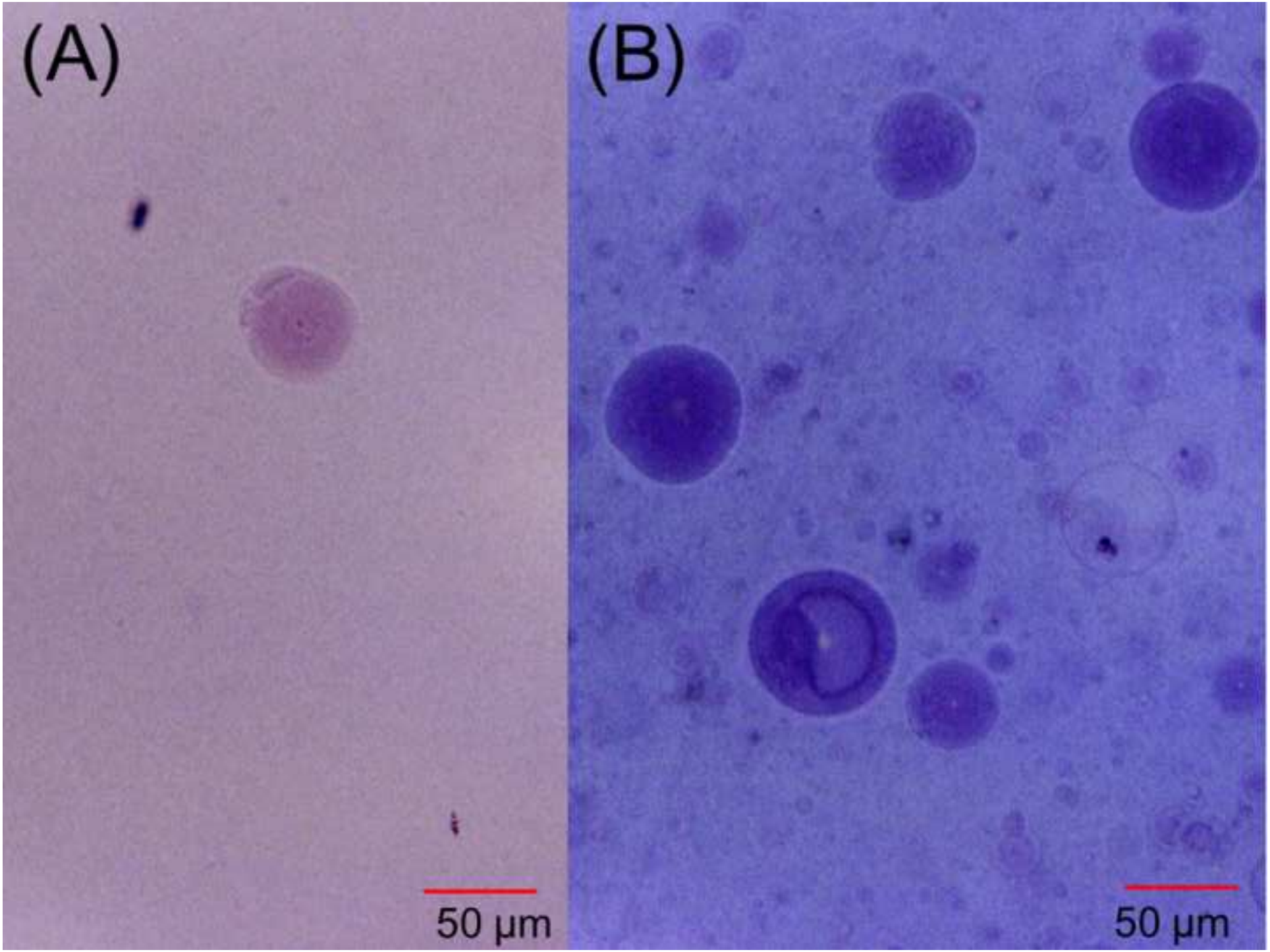

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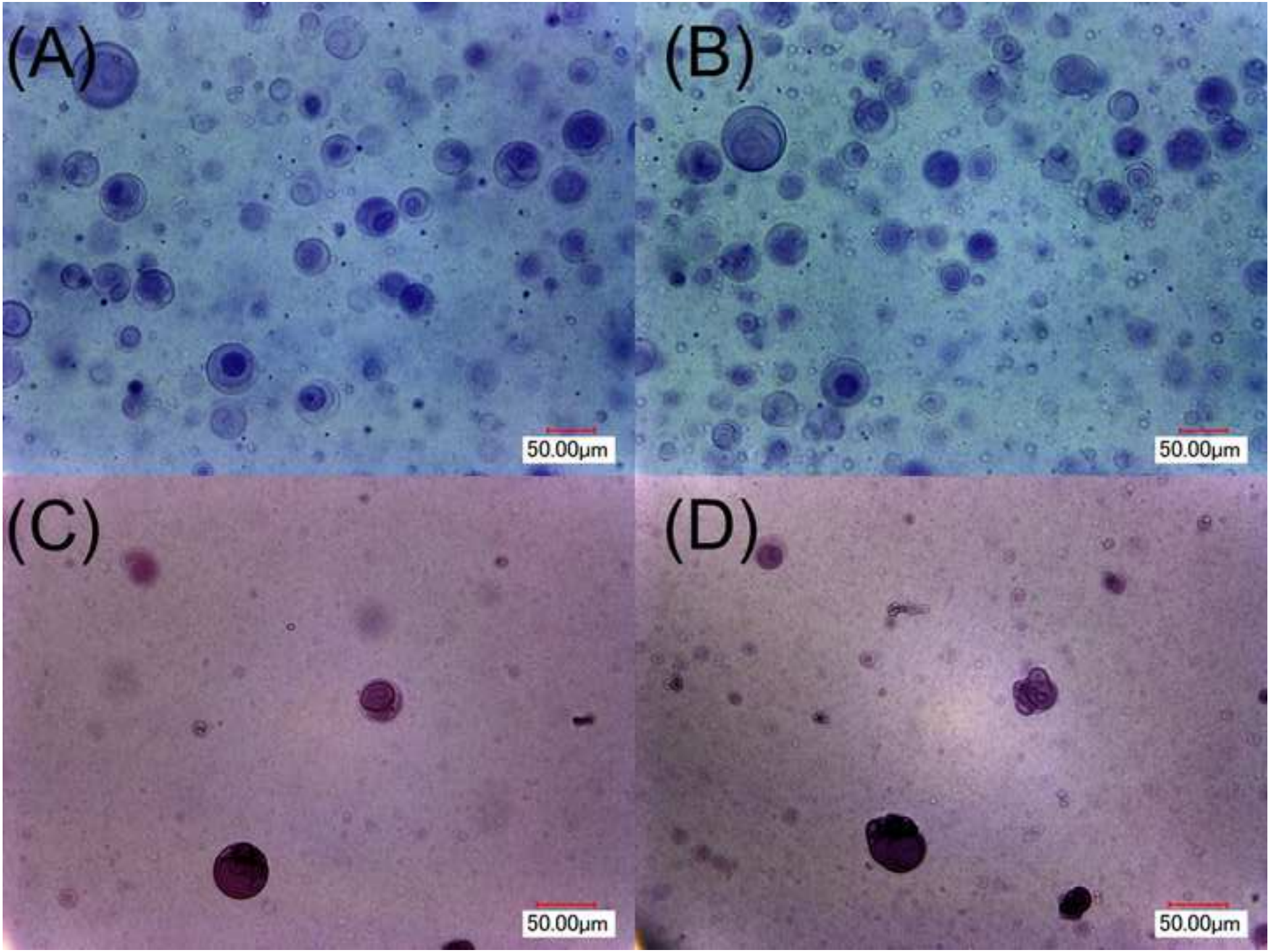

Figure 5

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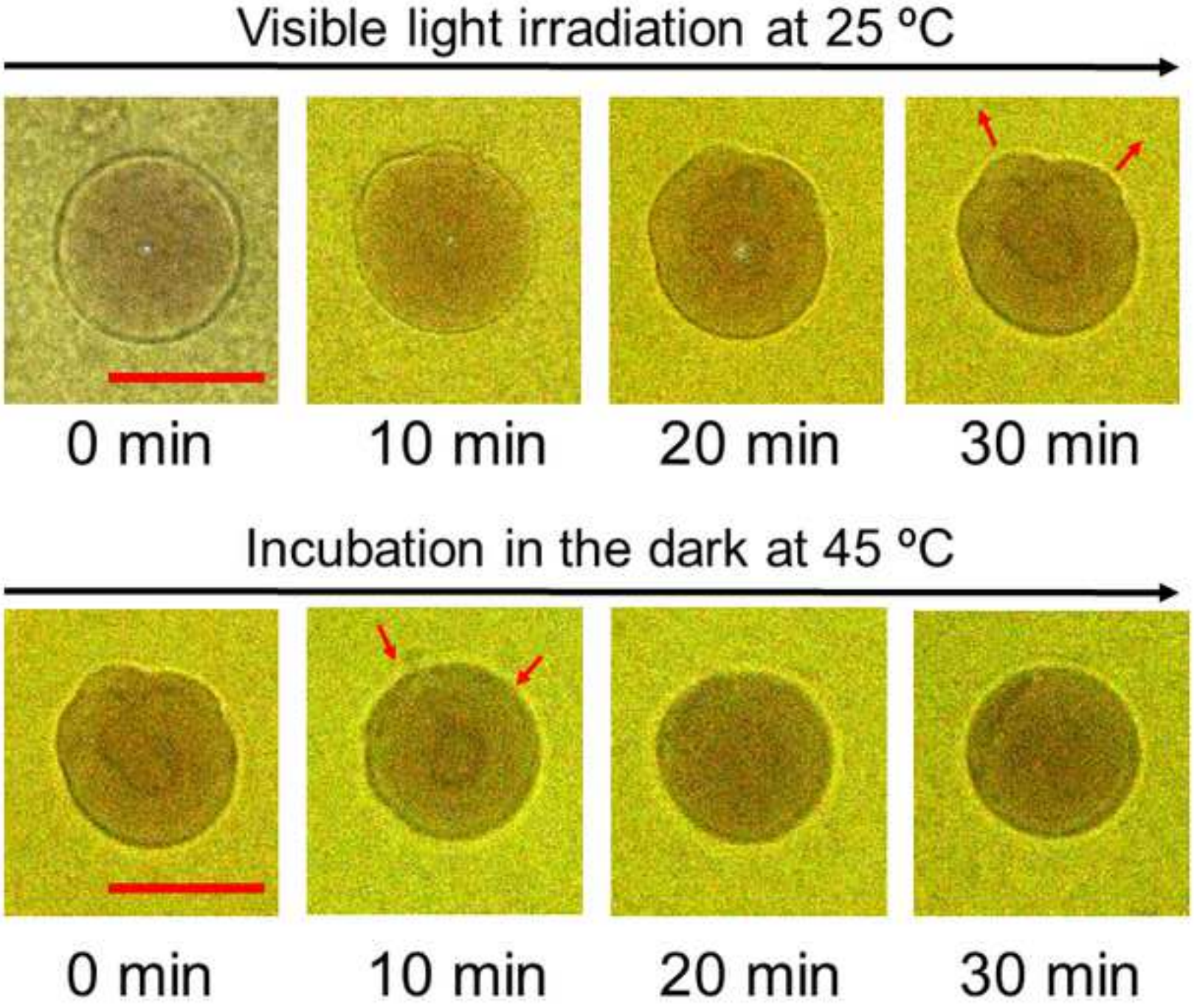

Figure 6

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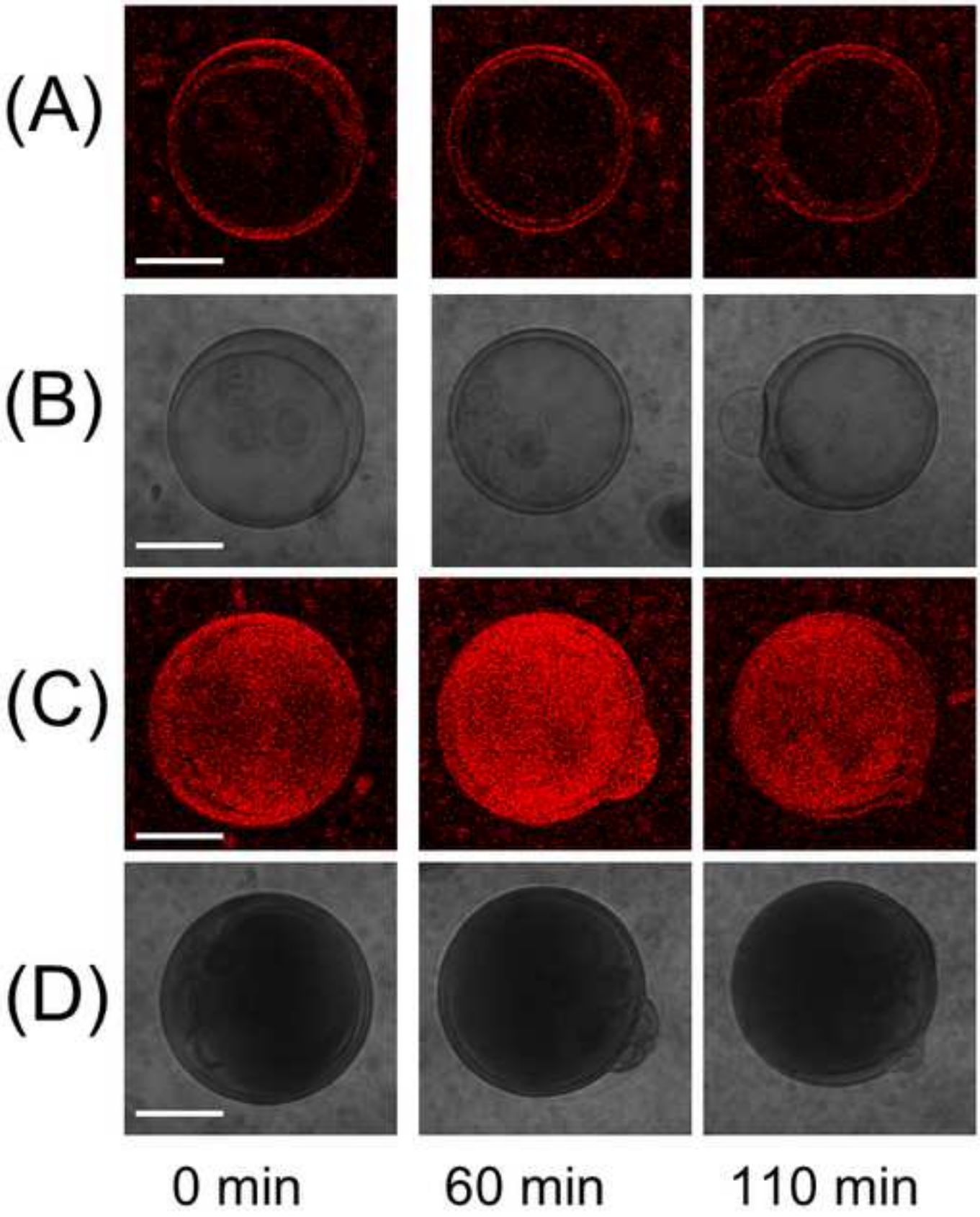

Table

Entry	Change from standard conditions[a]	Average size/ μm	Morphological change / %
1	none	15	79
2	*proximal*-**1**, 10 nmol	20	11
3	no *proximal*-**2**	24	8
4	*proximal*-**2**, 10 nmol	18	80
5	*proximal*-**2**, 5 nmol	22	33
6	DPPC	16	50
7	500 nmol Na_2SO_4	15	80
8	1000 nmol NaOH	21	10
9	100 nmol NaOH	27	27

[a] In standard conditions, vesicles are prepared from DOPC (100 nmol), *proximal*-**2** (20 nmol, 20 mol%), and water (100 μL).

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Triethylamine	Wako Pure Chemical Industries, Ltd.	202-02646	
Lithium Chloride	Wako Pure Chemical Industries, Ltd.	125-01161	
Chloroform	Kanto Chemical Co. Ltd.	07278-03	Used for vesicle preparation
Chloroform	Junsei Chemical Co. Ltd.	28560-0382	Used for ligand synthesis
Acetone	Junsei Chemical Co. Ltd.	11265-0382	
Ethanol	Junsei Chemical Co. Ltd.	17065-0382	
Ethyl Acetate	Junsei Chemical Co. Ltd.	67150-0382	
Hexane	Junsei Chemical Co. Ltd.	31055-0382	
Silica gel	Kanto Chemical Co. Ltd.	37558-79	100-210 μm
1-decanol	Tokyo Chemical Industry Co., Ltd.	D0031	25 mL
Potassium hydroxide	Kanto Chemical Co. Ltd.	32344-00	
Sodium hydrixude	Wako Pure Chemical Industries, Ltd.	197-02125	
Dimethyl sulfoxide (DMSO)	Kanto Chemical Co. Ltd.	10378-00	
d-DMSO	Sigma-Aldrich	166290100	
CD_3OD	Kanto Chemical Co. Ltd.	25221-43	
d-Acetone	Kanto Chemical Co. Ltd.	01054-43	
D_2O	Cambridge Isotope Laboratories, Inc.	DLM-4-100	
Ruthenium chloride n-Hydrate	Wako Pure Chemical Industries, Ltd.	183-00823	
2,2':6',2''-Terpyridine	Sigma-Aldrich	234672-5G	
0.1 mol/L Silver nitrate solution	Wako Pure Chemical Industries, Ltd.	192-00855	
Sodium sulfate	Kanto Chemical Co. Ltd.	37280-00	

1,2 Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)	Wako Pure Chemical Industries, Ltd.	160-12781	100 mg, stored at -20°C
1,2 Dioleoyl-sn-glycero-3-phosphocholine (DOPC)	Sigma-Aldrich	P6354-100mg	100 mg, stored at -20°C
1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein) (ammonium salt)	Avanti Polar Lipids, Inc.	Avanti 810332p	5 mg, stored at -20°C
1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)	Avanti Polar Lipids, Inc.	Avanti 810150c	1 mg, stored at -20°C
Dextran gel	GE healthcare Japan	17009010	Sephadex LH-20
Amber glass vial	Maruemu	0407-06	
Septum	Sigma-Aldrich	Z564648-100EA	
Heater	Advantech	DRM 320 DB	
Silicon film	AS ONE	6-9085-03	Thickness: 0.2 mm
Slide glass	Matsunami	S003130	76×26 mm, thickness: 0.8-1.0 mm
Cover glass	Matsunami	C218181	18×18 mm, thickness: 0.12-0.17 mm
Transfer pipette	Brand GMBH	704774	
Round-bottom flask	Vidtech	1500-05	
Sonicator	AS ONE	1-4591-32	
Optical power meter	OPHIR	ORION/PD P/N 1Z01803	
Oil bath	Riko	MH-3D	
Magnetic stirrer	Riko	MSR-10	
Diatomite	Wako Pure Chemical Industries, Ltd.	537-02305	Celite 545

Evaporator	Yamato	RE-52	
Glass funnel	Kiriyama	SB-21	10 mL, 21 mmφ
Bell jar	Kiriyama	VKB-200	
Filter paper	Kiriyama	No.4	21 mmφ
Optical microscope	KEYENCE	VHX-5000	
Confocal fluorescence microscope	Olympus	FV-1000	

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Author(s): Masanari Hirahara, Akira Tsukamoto, Hiroki Goto, Shigeru Tada, Masayuki Yagi, Yasushi Umemura

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Thank you very much for your kind comments. We have revised the manuscript carefully considering your comments. We hope that following replies meet your comments and we would be glad to respond to any further questions and comments that you may have.

Comments from Reviewers #3:

Comments:

Manuscript Summary: The authors have provided protocols for preparation of ruthenium based surfactants followed by their preparation/incorporation into DOPC and DPPC based giant vesicles. It was also demonstrated that fluorescein-DOPC are not suitable for monitoring the morphological changes of vesicles (absence of emission due to metal-ligand charge transfer), whereas rhodamine-DOPC were suitable for this.

Major Concerns:

1). The change in morphology suggested by the authors is not significant visually.

3). The multi-lamellar architecture claimed by authors cannot be seen visually from the figures. It would help if these changes are clearly labelled in the figures/pictures.

Answer: As you suggested, morphological changes in Figure 2 is somewhat unclear. There were 30 pictures in the original version in Figure 2. In the revised manuscript, we simplified figure 2 and 6 by omitting the several figures. We have added arrows indicating the morphological changes (budding, size change) in the figures. Also, the font size in the figures was enlarged.

2). The authors claim that morphology change is seen only in the presence of Na2SO4 and not in NaOH, figures of this claim need to be shown, and brief discussion of the reason for this effect.

Answer: We have added Figure 4 showing the vesicles prepared in an aqueous solution of Na_2SO_4 or NaOH and their morphological changes under visible light irradiation. Short discussion was added on the differences of the two vesicle dispersions.

4). The labeling of Figure 2 is unclear. Are figures, 2A, 2B, and 2C from same solution? why do they all have different contrasts?

Answer: Vesicles in Figure 2A-2C were obtained from the same dispersions. As you suggested, contrasts between 0 min and 16 min are different. This is because we tuned white balance of microscope during the measurements in order to see the interior lamellar structures of vesicles shown in figure 2A.

Comments from Reviewers #4:

Comments:

Manuscript Summary: In this paper, the authors have shown that giant vesicles can be fabricated from photo-responsive ruthenium complex. The resultant vesicles can undergo the morphological change after being exposed to visible light due to the photo-isomerization. The experiments were well designed and data are sufficient to support the conclusion. I recommend this work to be published in JVE.

Additional Comments to Authors:

Also, It will be interesting to see if this morphological change can be also reversible due to the reversible photo-isomerization which presumably changes the critical packing shape of surfactants back and forth.

Answer: In a previous article, we reported that the surfactant *proximal*-**2** showed photoisomerization equilibrium between the distal isomer, and the mixture of *proximal* and *distal*-**2** in the photostationary state showed back isomerization to *proximal*-**2** at 45 °C in a mixed aqueous solution in the dark. In the revised version, we carried out photo- and thermal-induced morphological changes of the vesicles. The results of the experiments were added in Figure 5 and brief discussion was added.

Reply to editorial comments

Thank you for fruitful comments on the manuscript. We carefully edited the revised manuscript following the editorial comments. Through the revision of manuscript, the length of the protocol exceeded the limit. Please use the highlighted part for the video article.

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All of your previous revisions have been incorporated into the most recent version of the manuscript. In addition, Editor may have made minor copy edits to your manuscript and formatting changes to comply with the JoVE format. Please maintain these changes. On the JoVE submission site, you can find the updated manuscript under "file inventory" and download the microsoft word document. ***Please use this updated version for any future revisions and track all changes using the track changes function in Microsoft Word****.*

•Commercial language (brand names of microscopes) was removed from the manuscript. All commercial products should be sufficiently referenced in the Table of Materials.

•Formatting:
-Please include spaces between all paragraphs and bullet points.
-Please include spaces between numbers and units.
-Please revise the legend for figure 1 so that it has a separate title (in bold) followed by a short description of the figure.
-References – Please abbreviate all journal titles.

•Grammar:
-Section 1 heading – "Syntheses" should be "Synthesis" for a single chemical.
-4.3 – Should be "hole punch"
-4.7 – "Cover with the vesicle dispersion with a cover glass"
-Line 270 – "Common troubleshooting for the protocols are"

•Additional detail is required:
-1.4, 2.2.5 – Please provide a citation for chromatography as insufficient detail is provided.
-1.5 – How is the blue band identified/collected? Is it in fractions eluted from the column? Please provide a literature reference for silica gel chromatography.
-2.2.5 – How is the purple band identified/collected? Is it in fractions eluted from the column? For TLC, what is the stationary phase? How much sample is spotted on the TLC plate? Is the product visualized in some way?

-1.6, 2.2.6, 2.3.5 – please provide literature reference(s) for 1H and 13C NMR.
-3.8 – What is one checking for?
-4.4 – Wipe with what?
-4.5 – How is it attached? Is it simply laid on the plate? Are bubbles avoided?
-5.3 – How far from the plate is the lamp?
-6.4 – What is the intensity adjusted to?

•Prior to peer review, the protocol length is at our 3 page limit. If additional details are added to the protocol, please use yellow highlighting to identify a total of 2.75 pages of protocol text (which includes headings and spaces) that should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification and remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

•Discussion: Please discuss the significance of the technique with respect to alternative methods. Please also discuss the limitations and future applications of the method.

•Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

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