Electronic supplementary information

Protein-coated nanocapsules via multilevel surface modification. Controlled preparation and microscopic analysis at nanometer resolution

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Experimental section

General.

All reactions dealing with air- or moisture-sensitive compounds were carried out in a dry reaction vessel under nitrogen or argon. The water content of the solvent was confirmed with a Karl-Fischer Moisture Titrator (MKC-210, Kyoto Electronics Company) to be less than 10 ppm. Analytical thin-layer chromatography (TLC) was performed on glass plates coated with 0.25 mm 230–400 mesh silica gel containing a fluorescent indicator (Merck). Analysis with high performance liquid chromatography (HPLC) was performed on JASCO HPLC system equipped with a Bucky Prep column (Nacalai Tesque Cosmosil Buckyprep, 4.6 × 250 mm; column temperature at 40 °C). Flash silica gel column chromatography was performed on silica gel 60N (Kanto, spherical and neutral, 140–325 mesh) as described by Still *et al*.[1] Gel permeation column chromatography was performed on a Japan Analytical Industry LC-908 (eluent:

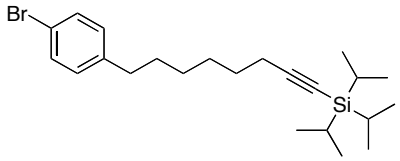
chloroform) with JAIGEL 1H and 2H polystyrene columns. Nuclear magnetic resonance (NMR) spectra were measured on JEOL ECX-400 and ECA-500 spectrometers and reported in parts per million from tetramethylsilane. ^{1}H NMR spectra in $CDCl_3$ were referenced internally to tetramethylsilane as a standard, and ^{13}C NMR spectra to the solvent resonance. (Methyl, methylene, and methyne signals in ^{13}C NMR spectra were assigned by DEPT spectra. High resolution mass spectra were acquired by atmospheric pressure chemical ionization (APCI) using a time-of-flight mass analyzer on a JEOL AccuTOF JMS-T100LC spectrometer with a calibration standard of polyethylene glycol (MW 1000). Distilled water was further deionized with Millipore Milli-Q. Dynamic laser light scattering (DLS) was carried out on a Malvern Zetasizer Nano ZS machine. Atomic force microscopy (AFM) was performed on a Bruker MultiMode 8. Scanning electron microscopy (SEM) with energy dispersive X-ray analysis (EDX) was performed on a FEI Magellan 400L equipped with AMETEK/EDAX Genesis APEX4. Scanning transmission electron microscopy (STEM) was performed on a JEOL JEM-2100F. The UV-Visible spectra were recorded on a JASCO V-570 UV/Vis/NIR Spectrophotometer. Fluorescence spectra were recorded on a HITACHI F-4500 Fluorescence Spectrophotometer.

Materials.

Unless otherwise noted, materials were purchased from Tokyo Kasei Co., Aldrich Inc., and other commercial suppliers and used after appropriate purification before use. Anhydrous ethereal solvents (stabilizer-free) were purchased from WAKO Pure Chemical and purified by a solvent purification system (GlassContour)[2] equipped with columns of activated alumina and supported copper catalyst (Q-5) prior to use. All other solvents were purified by distillation and stored over molecular sieves 4Å. Synthesis of reported compounds 1-Bromo-4-(6-iodohexyl)benzene,[3] and 7-hydroxy-3-azidocoumarin

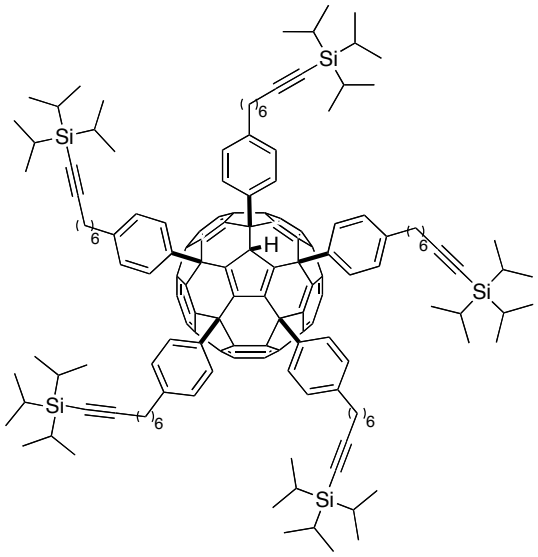
1[4] were carried out following the literatures.

[8-(4-Bromophenyl)oct-1-ynyl]tri(1-methylethyl)silane



1.60 M of *n*-Butyl lithium in hexane (3.74 mL, 5.99 mmol; 2.2 equiv.) was added dropwise to a solution of triisopropylacetylene (1.00 g, 5.49 mmol; 2.0 equiv.) in THF (4.0 mL) at 0 °C. After stirring for 30 min, the reaction mixture was added dropwise to a solution of 1-(4-bromophenyl)-6-iodohexane (996 mg, 2.71 mmol) in 1,3-dimethyltetrahydro-2(1*H*)-pyrimidone (4.0 mL) at 0 °C. The reaction was monitored by TLC. After stirring for 70 min, the reaction mixture was poured into ice-cold water. The organic layer was extracted three times with pentane (20 mL). The combined extracts were washed twice with brine (15 mL), dried over anhydrous magnesium sulfate, and concentrated under reduced pressure. The crude material (1.68 g) was purified by flash column chromatography on silica gel (85 g, eluent: hexane) to give the title compound (830 mg, 73%) as a pale yellow oil: IR (neat) 2936, 2826, 2172, 1488, 1462, 1243, 1072, 1012, 995, 919, 883, 797, 676, 661, 622 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.88–1.09 (m, 21H), 1.29–1.36 (m, 2H), 1.41–1.62 (m, 6H), 2.24 (t, *J* = 6.9 Hz, 2H), 2.55 (t, *J* = 7.5 Hz, 2H), 7.04 (d, *J* = 8.6 Hz, 2H), 7.38 (d, *J* = 8.6 Hz, 2H); ^{13}C NMR (125 Hz, $CDCl_3$) δ 11.30, 18.64, 19.78, 28.42, 28.55, 28.68, 31.18, 35.21, 64.38, 80.13, 109.11, 130.15, 131.25, 141.69; Anal. Calcd for $C_{21}H_{37}BrSi$: C, 65.53; H, 8.85. Found: C, 65.53; H, 8.95.

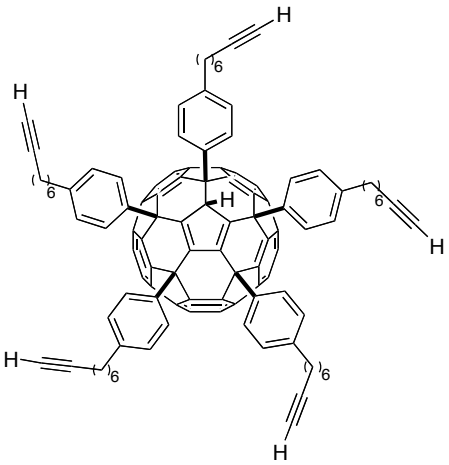
6,9,12,15,18-Penta(4-{8-[tri(1-methylethyl)silyl]oct-7-ynyl}-phenyl)-1,6,9,12,15,18-hexahydro(C_{60}-I_h)[5,6]fullerene (C8(7Y8TIPS)H)



0.16 M of 4-[8-(triisopropyl)oct-7-ynyl]phenyl magnesium bromide in THF (8.0 mL, 1.90 mmol, 0.23 M; 12 equiv.), prepared from [8-(4-bromophenyl)oct-1-ynyl]tri(1-methylethyl)silane, magnesium and catalytic amount of 1,2-dibromoethane, was added to a solution of CuBr•SMe_2 (390 mg, 1.90 mmol; 12 equiv.) in THF (1.0 mL) at 35 °C. After stirring for 10 min, a solution of [60]fullerene (120 mg, 0.158 mmol) in 1,2-dichlorobenzene (8 mL) was added to the reaction mixture. The reaction was monitored by HPLC (Buckyprep, eluent: 30% 2-propanol/toluene). After stirring for 50 min, the reaction was quenched with sat. aq. NH_4Cl (0.5 mL). The reaction mixture was filtrated through a pad of silica gel eluted with carbon disulfide to remove copper salts, and then was concentrated under reduced pressure. Since isolation of the target compound by reprecipitation and chromatographic methods were unsuccessful due to low crystallinity and polarity, the crude material containing 1,2-dichlorobenzene (9.55 g) was used for the next deprotection reaction without further purifications: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.95–1.08 (m, 105H), 1.20–1.67 (m, 40 H), 2.22–2.28 (m, 10H), 2.54 (t, J = 7.5 Hz, 2H), 2.59 (t, J = 7.5 Hz, 4H), 2.65 (t, J = 7.5 Hz, 4H), 5.24 (s, 1H), 6.94 (d, J = 8.0 Hz, 2H), 6.98 (d, J = 8.0 Hz,

4H), 7.11 (d, *J* = 8.0 Hz, 4H), 7.32 (d, *J* = 8.0 Hz, 2H), 7.49 (d, *J* = 8.0 Hz, 4H), 7.66 (d, *J* = 8.0 Hz, 4H).

6,9,12,15,18-Penta[4-(oct-7-ynyl)phenyl]-1,6,9,12,15,18-hexahydro-(C_{60}-I_h)[5,6]fullerene (C8(7Y)H)



1.00 M of Bu_4NF in THF (1.58 mL, 1.58 mmol, 1.0 M; 10 equiv.) was added to a solution of crude material of 6,9,12,15,18-penta{4-[(8-triisopropylsilyl)oct-7-ynyl]-phenyl}-1,6,9,12,15,18-hexahydro(C_{60}-I_h)[5,6]fullerene **C8(7Y8TIPS)H** in THF (10 mL), and the reaction mixture was started refluxing. The reaction was monitored by APCI-TOF mass (negative ion mode). After stirring for 1.5 h, the reaction mixture was slowly added into methanol (50 mL) to obtain orange precipitate. The precipitate was collected by filtration, washed with methanol, and dried under reduced pressure to give the title compound (170 mg, 66% in 2 steps) as an orange solid: IR (powder) 2929, 2856, 2363, 2342, 1719, 1509, 1093, 1017, 625 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 1.25–1.70 (m, 40H), 1.92–1.96 (m, 5H), 2.14–2.22 (m, 10H), 2.54 (t, *J* = 7.5 Hz, 2H), 2.59 (t, *J* = 7.5 Hz, 4H), 2.65 (t, *J* = 7.5 Hz, 4H), 5.23 (s, 1H), 6.92 (d, *J* = 8.6 Hz, 2H), 6.97 (d, *J* = 8.6 Hz, 4H), 7.11 (d, *J* = 8.6 Hz, 4H), 7.30 (d, *J* = 8.6 Hz, 2H), 7.48 (d, *J* = 8.6 Hz, 4H), 7.65 (d, *J* = 8.6 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 18.38, 18.43, 18.46, 28.40, 28.46, 28.46, 28.55, 28.56, 28.58, 28.60,

28.63, 28.67, 31.18, 31.25, 31.26, 35.32, 35.36, 35.40, 58.64, 58.72, 60.77, 62.97, 68.20, 68.24, 68.25, 77.21, 84.59, 84.62, 127.70, 127.99, 128.10, 128.19, 128.37, 128.57, 128.65, 128.83, 137.18, 137.24, 141.55, 141.80, 142.07, 143.08, 143.19, 143.86, 144.00, 144.08, 144.20, 144.29, 144.31, 144.55, 145.48, 145.75, 146.00, 146.27, 146.87, 147.06, 147.17, 147.69, 147.98, 148.03, 148.21, 148.35, 148.63, 148.69, 151.81, 152.40, 152.56, 156.26; HRMS (APCI+) calcd for $C_{130}H_{86}^{+}$ $[M]^{+}$ 1648.6842, found 1648.6823.

Preparation of C8(7Y)K vesicle

The vesicle solution used for this study was prepared as reported:[5] *t*BuOK in THF (1.00 M, 74 μL, 75 μmol, 1.5 equiv.) was added to a solution of **C8(7Y)H** (82.4 mg, 50 μmol) in THF (3.93 mL) and mixture was stirred under argon. After stirring for 3 h, a portion of the solution of **C8(7Y)K** (12.5 mM, 0.80 mL, 10 μmol) was slowly injected into ultrapure water (4.2 mL) with stirring at 400 rpm over 1 min using a syringe pump (ISIS Co.) to obtain a vesicle solution of **C8(7Y)K** (2.0 mM) in 16% THF/water. THF and water were removed by evaporation at ca. 7 kPa, and the final concentration of **C8(7Y)K** was adjusted to 2.0 mM. If it is necessary to obtain vesicle solution with narrower size distribution, the vesicle solution was centrifuged (TOMY MC-150) at 15,000 rpm for 30 min, and the supernatant was collected. The concentration of **C8(7Y)K** was determined to be 1.5 mM by UV spectra at 352 nm of wavelength. The diameter of the vesicles may vary in the range of 18–32 nm, depending on the conditions of the preparation.

Fluorescence analysis of Cu(I)-catalyzed Huisgen [3 + 2] cycloaddition reaction of azido-coumarin 1

To estimate the efficiency of surface modification on the **C8(7Y)K** vesicles by the click reaction, we first examined the copper(I)-catalyzed click reaction with an azidocoumarin **1** (Figure S1a), which by itself is fluorescently

inactive but becomes intensely fluorescent after the formation of 1,2,3-triazole-linked coumarin because of the loss of the azido group by cycloaddition. We can monitor the reaction by the fluorescence of the 7-hydroxycoumarin group at 530 nm (λ_{ex} = 490 nm) even at a micromolar concentration.

We confirmed in a separate experiment that **biotin-N_3** also reacts with 1-hexyne with much the same efficiency as azidocoumarin **1**. Hence the 19% yield determined for **1** (see below) should be similar for **biotin-N_3**, and the number of biotin groups introduced on the vesicle surface that have ca. 2000 alkynyl terminals is estimated to be ca. 400. The ca. 5-nm size of avidin permits only ca. 60 molecules to be attached to the 16-nm-sized vesicle, hence the 400 biotin groups are enough to cover the vesicles surface with avidin molecules.

Stock solutions of 7-hydroxy-3-aidocoumarin **1** (2.5 mM), copper(II) sulfate (0.40 mM), (+)-sodium ascorbate (0.80 mM), and triethylamine (4.0 mM) was prepared in ultrapure water. 2.0 mM of **C8(7Y)K** or **C8K**[5] (a saturated analogue of **C8(7Y)K**) vesicle in water (2.5 μL) was added to a solution of 7-hydroxy-3-azidocoumarin **1** (10.0 μL, 25 nmol; 5.0 equiv.), copper(II) sulfate 6.25 μL, 2.5 nmol; 50 mol%), (+)-sodium ascorbate (6.25 μL, 5.0 nmol; 1.0 equiv.) and triethylamine (6.25 μL, 25 nmol; 5.0 equiv.) in distilled-deionized water (5.0 mL) at room temperature in a 1.0 × 1.0 x 4.0 cm^3 quartz cuvette (Figure S1). Time-dependent fluorescent intensities were measured at excitation wavelength of 490 nm at 25 °C, and monitored at emission wavelength of 530 nm with the slit width of 5 nm. The fluorescence intensity at 530 nm increased and reached a plateau after 12 days (Figure S1c red).

By comparison of the molar fluorescent intensity of a reference addition product of **1** and 1-hexyne (Figure S2) and with the (marginal) background decomposition of **1** under the same conditions using **C8K** (Figure

S1c blue), we estimate that 19% of the terminal alkyne groups (i.e., one octynyl group/one fullerene) reacted with the azidocoumarin **1**.

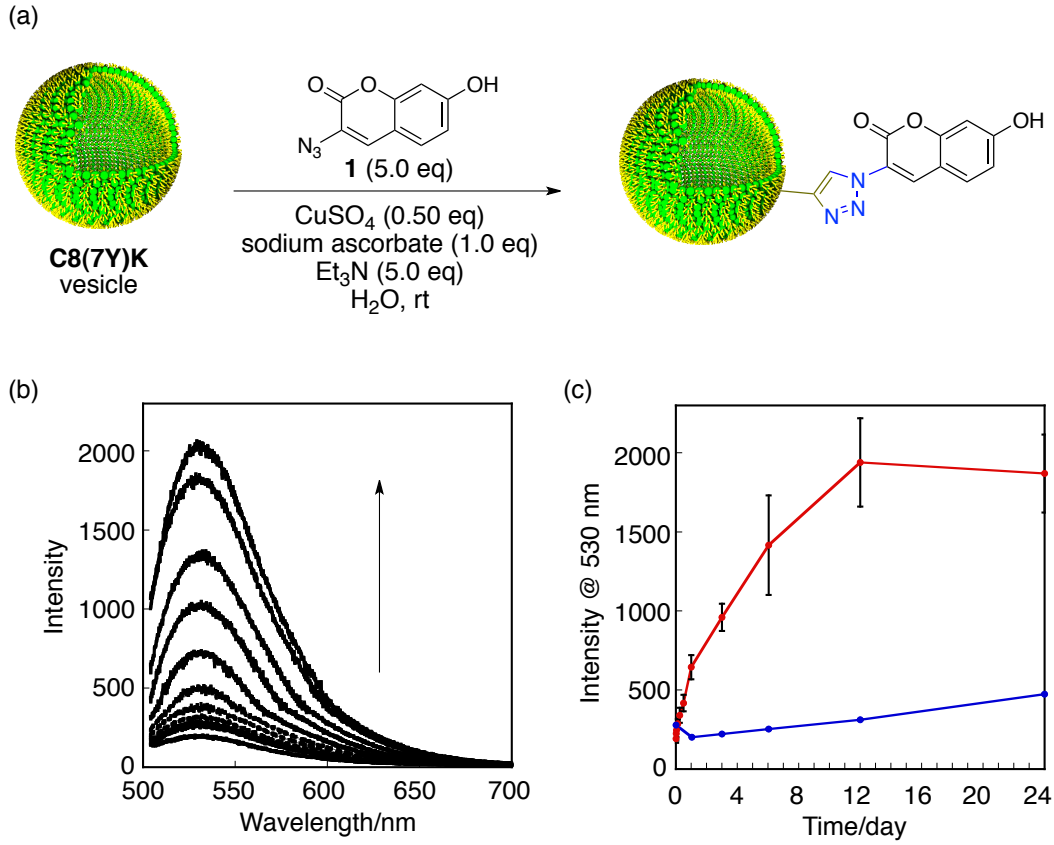


Figure S1. Fluorescent analysis of **C8(7Y)K** vesicle with azidocoumarin **1**. (a) Schematic image of click reaction with azidocoumarin **1** on the vesicle surface. (b) Fluorescent intensity of **C8(7Y)K** vesicle (λ_{ex} = 490 nm at 25 °C in water). (c) Fluorescent intensity at 530 nm for the **C8(7Y)K** vesicle (red), the **C8K** vesicle (blue) as a function of reaction time.

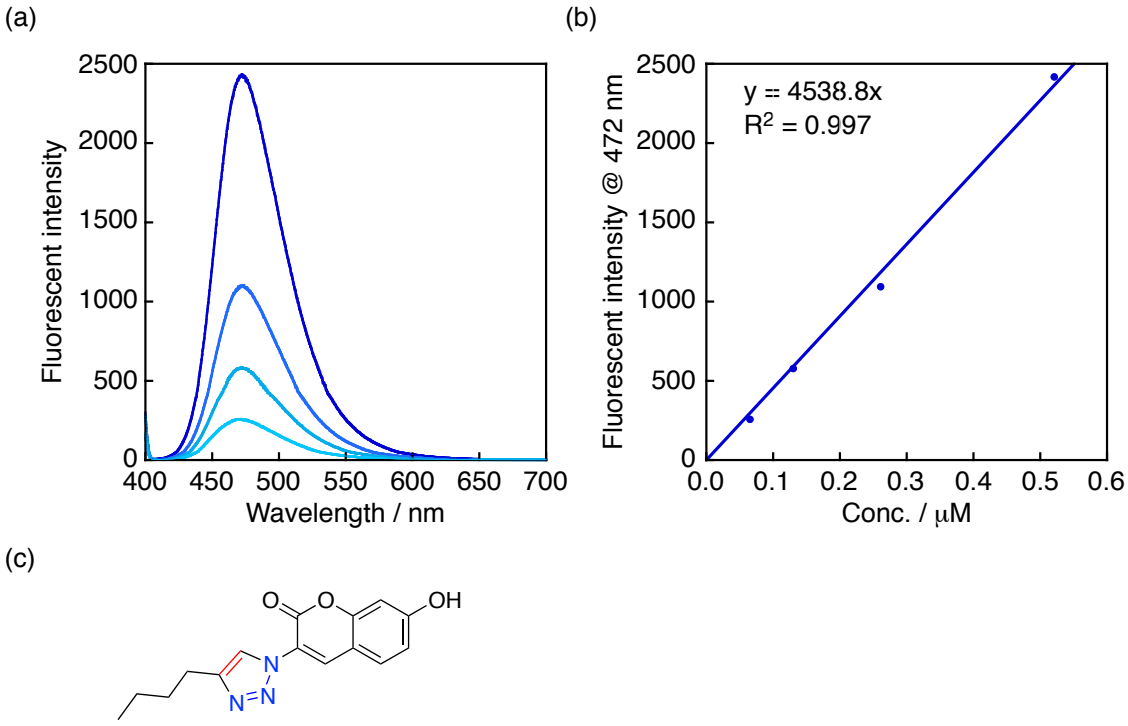
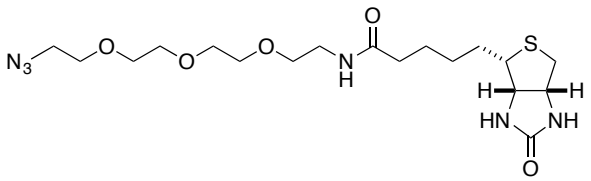


Figure S2. Fluorescent analysis of reference addition product of azidocoumarin **1** and 1-hexyne. (a) Fluorescent intensity (λ_{ex} = 390 nm at 25 °C in water) in the range of concentration from 0.07 to 1.04 μM. (b) Fluorescent intensity at 472 nm as a function of concentration. (c) Structure of a reference product.

Preparation of biotinylated C8(7Y)K vesicle

Biotinylated fullerene vesicle was prepared using Cu(I)-catalyzed Huisgen [3 + 2] cycloaddition reaction of azido-biotin with alkynyl fullerene vesicle. Stock solutions of **biotin-N$_3$** (0.10 mM), copper(II) sulfate (0.010 mM), (+)-sodium ascorbate (0.060 mM), and triethylamine (0.10 mM) in distilled-deionized water was prepared. Alkynyl fullerene vesicle (1.5 mM, 400 μL, 0.60 μmol) was added slowly with stirring to a solution of **biotin-N$_3$** (30 μL, 0.30 nmol; 5.0 equiv.), copper(II) sulfate (30 μL, 0.30 nmol; 0.50 equiv.), (+)-sodium ascorbate (10 μL, 0.60 nmol; 1.0 equiv.), and triethylamine (30 μL, 3.0 nmol; 5.0 equiv.) at room temperature. After stirring for 6 days, the reaction mixture was passed through a column of Sephadex G-50 fine (GE healthcare

Japan, Japan, ϕ 10 × 100 mm) to be separated with unreacted **biotin-N_3**, and orange-colored fractions in the eluted solution were collected and diluted with distilled-deionized water to 5.0 mL ([**C8(7Y)K**] = 0.15 mM). The size of biotinylated **C8(7Y)K** vesicle was determined by DLS analysis and SEM measurement.



biotin-N_3

Figure S3. Structure of **biotin-N_3**.

Preparation of avidin-coated vesicle

An aqueous solution of biotinylated **C8(7Y)K** vesicle (0.15 mM, 13.5 μL, 2.0 nmol) was added to avidin (1.9 nmol) in phosphate buffered saline (100 μL) and mixed gently by a pipette at room temperature for 1 min. The size of vesicle aggregates in solution was analyzed by DLS. For SEM measurement, the solution was diluted to 50 μM (as the concentration of **C8(7Y)K**) and subjected to SEM sample preparation described below.

DLS analyses

DLS analyses were performed on a Malvern Zetasizer Nano ZS equipped with a He-Ne laser operating at 4 mW power and 633 nm wavelength, and a computer-controlled correlator, at a 173° accumulation angle. Measurement was carried out in a polystyrene or glass cuvette. The **C8(7Y)K** vesicle was equilibrated for 2 min at the set temperature each time. The data were processed using Dispersion Technology software version 4.10 to give Z-average particle size and polydispersity index (PDI) value by cumulant analysis, and particle size distribution by CONTIN analysis (Figure S4a).[6]

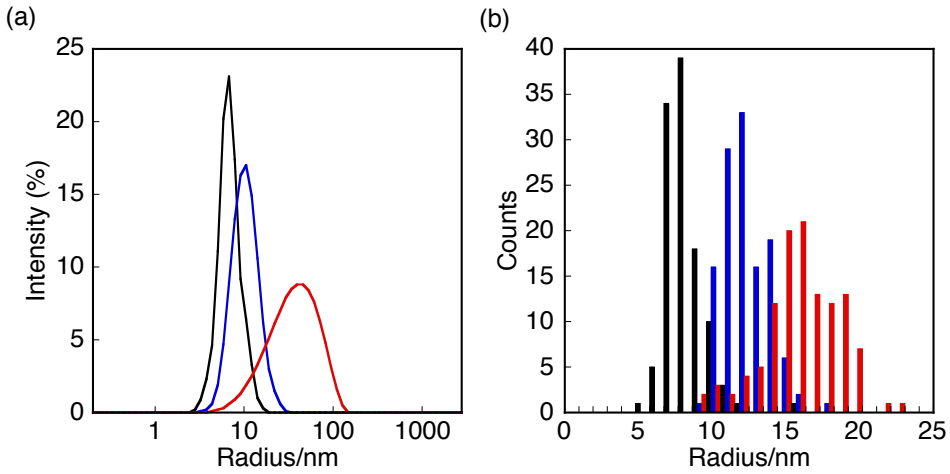


Figure S4. Comparison between the vesicle radii determined by DLS and by electron microscopy. (a) Hydrodynamic radii distribution of **C8(7Y)K** vesicles (black), biotinylated vesicles (blue) and avidin-covered vesicles (red) determined by DLS. (b) Histogram of the number of **C8(7Y)K** vesicles plotted against the radius of the vesicles. Black, **C8(7Y)K** vesicle (N = 112) observed from STEM images (Figure S5); Blue, Biotinylated **C8(7Y)K** vesicle (N = 123) observed from SEM images (Figure S6); Red, Avidin-coated vesicle (N = 116) observed from SEM images (Figure S7). Same color coding as in Figure S4a.

STEM measurement

STEM measurement was conducted on a JEOL JEM-2100F at 294 K with a spherical aberration coefficient Cs = 1.0 nm at an acceleration voltage of 200 kV under reduced pressure of 1.0×10^{-5} Pa in the sample column. The current density is ca. 0.5 pA·cm^{-2}. The imaging instrument used was an ultrascan charge-coupled device (CCD) camera (512 × 512 pixels). 50 μM of **C8(7Y)K** vesicle (2 μL) was deposited on a transmission electron microscopy (TEM) copper mesh coated with carbon film (Super Ultra High Resoluton Carbon film, thickness < 6 nm, Oken Shoji Co., Ltd.), then dried under reduced pressure (4×10^{-2} Pa) at room temperature for 18 h.

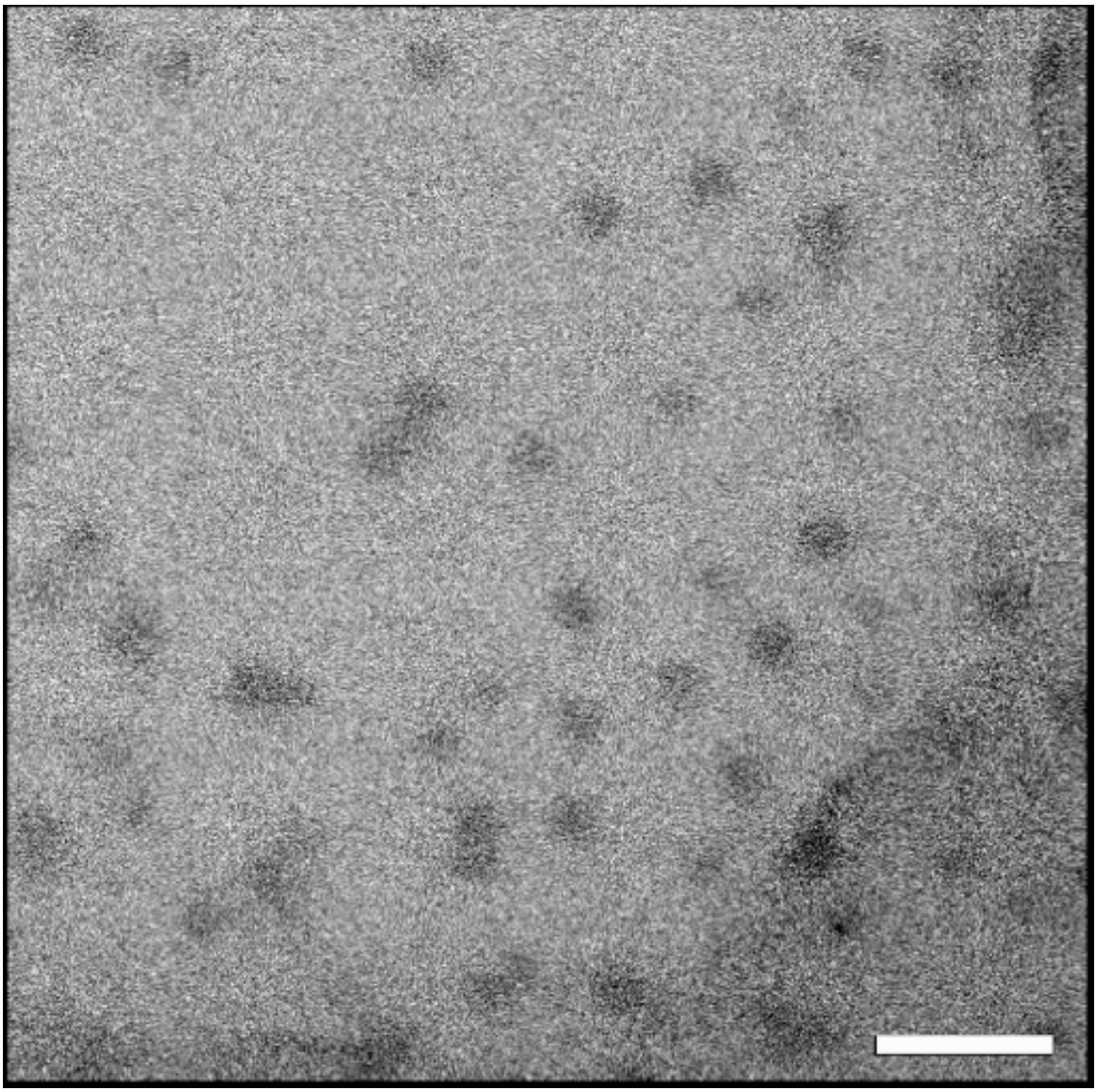


Figure S5. STEM image of **C8(7Y)K** vesicle on a carbon film without staining. Scale bar is 50 nm. Distribution of vesicle radius is shown in Figure S4b, black.

SEM measurement

SEM measurement was conducted on a FEI Magellan 400L. An aqueous solution of the vesicles (50 μM, 20 μL) was placed on an ITO/glass substrate cleaned by UV/ozone treatment just before use, and was spin-coated (1500 rpm) for 30 s. After drying under reduced pressure (5×10^{-2} Pa) for 10 min, the ITO substrate was subjected to the SEM observation at an acceleration voltage of 1–8 kV under a vacuum of 5×10^{-5} Pa without any conductive

coatings. We took most of the images at low acceleration voltage of 1–2 kV for higher resolution (Figures Figure S7 and Figure S8), but when the size of the vesicles were small and comparable to the size of ITO grains, we applied higher acceleration voltage of 8 kV so that the spherical shape of the small vesicle particles were highlighted obviously (Figure S6).

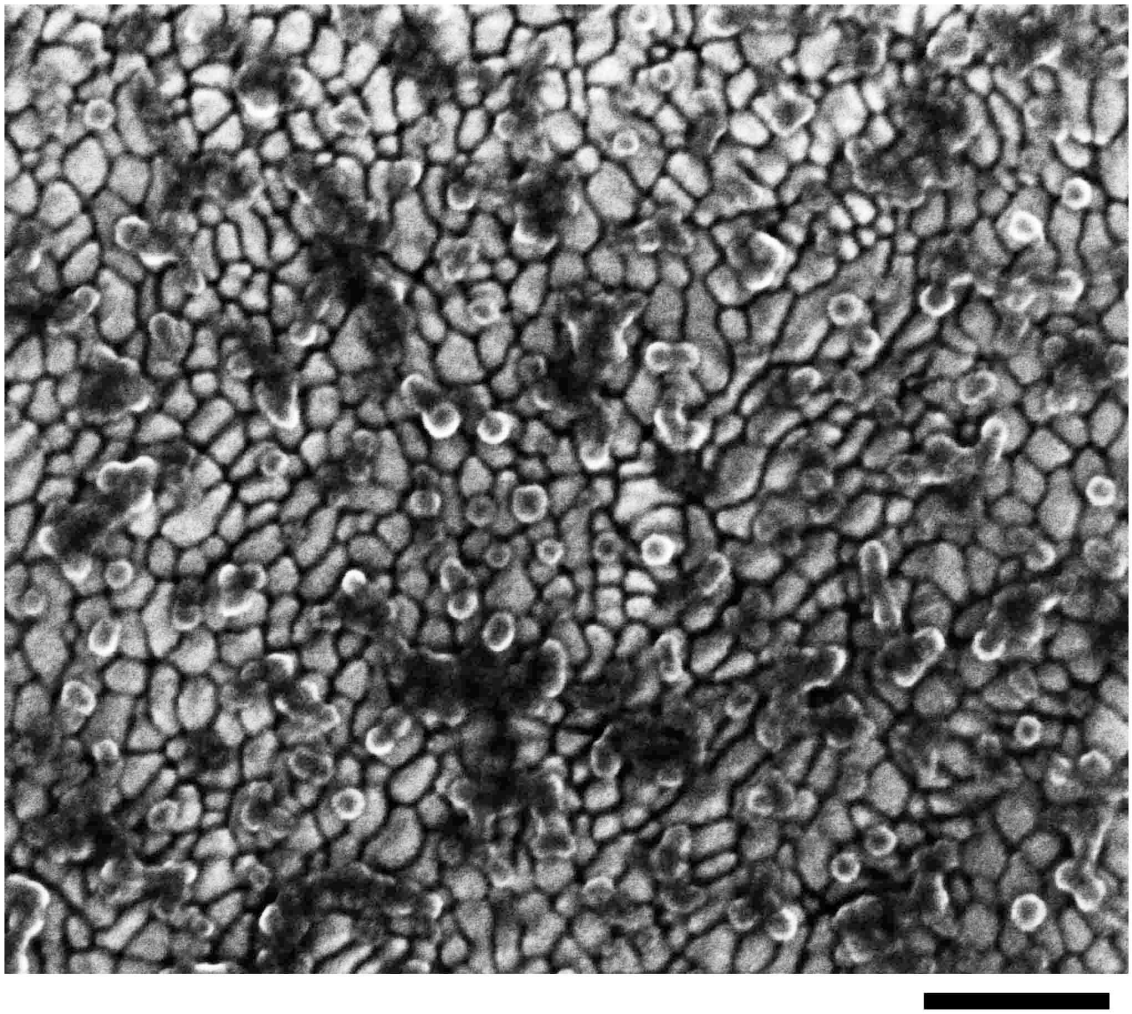


Figure S6. SEM image of biotinylated **C8(7Y)K** vesicle on ITO taken at acceleration voltage of 8 kV and current intensity of 50 pA. Scale bar is 100 nm. Distribution of vesicle radius is shown in Figure S4b, blue.

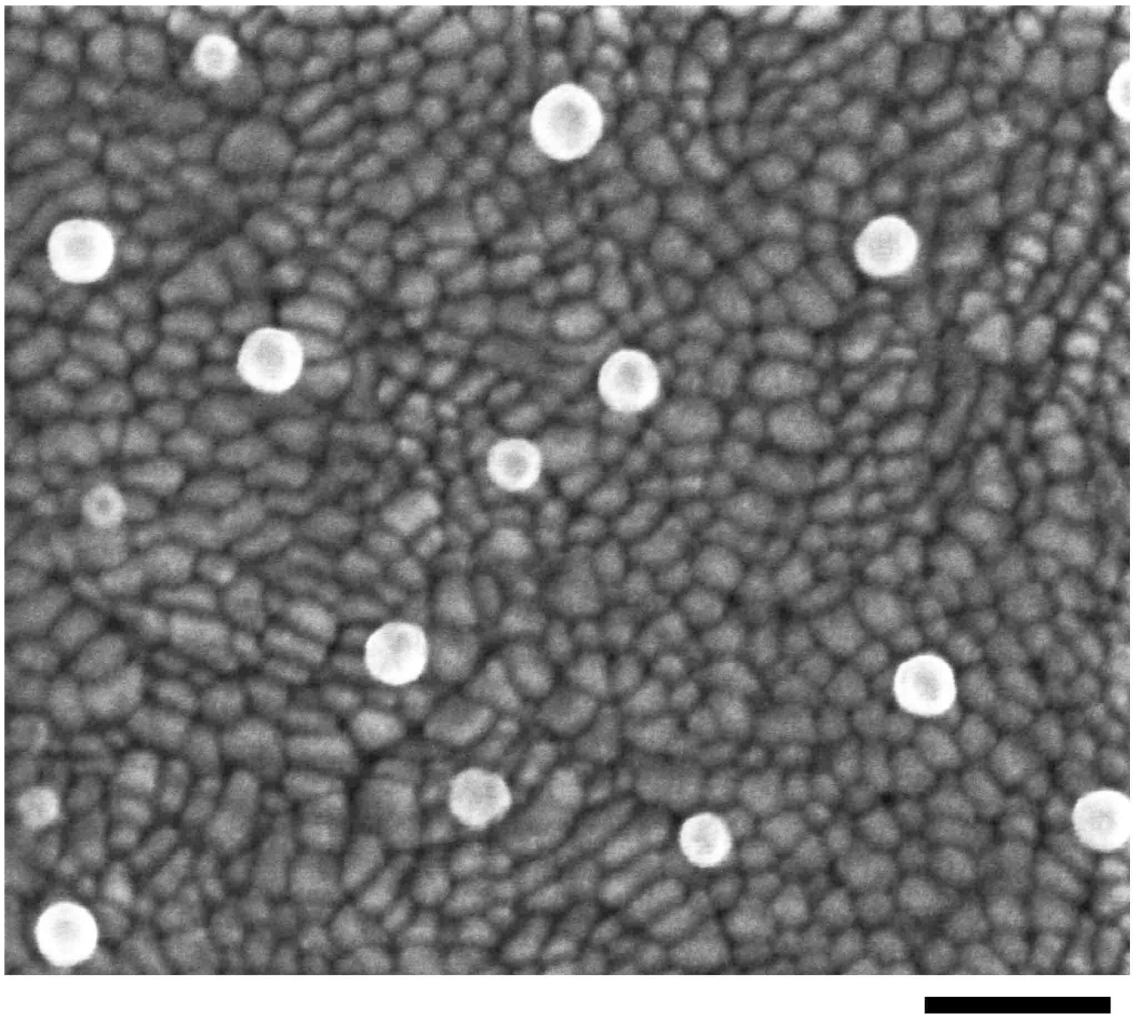


Figure S7. SEM image of avidin-coated vesicles on ITO taken at acceleration voltage of 2 kV and current intensity of 25 pA. This image was obtained from the first scan of this area (i.e. least effect on the specimen by electron irradiation). Scale bar is 100 nm. Distribution of vesicle radius is shown in Figure S4b, red.

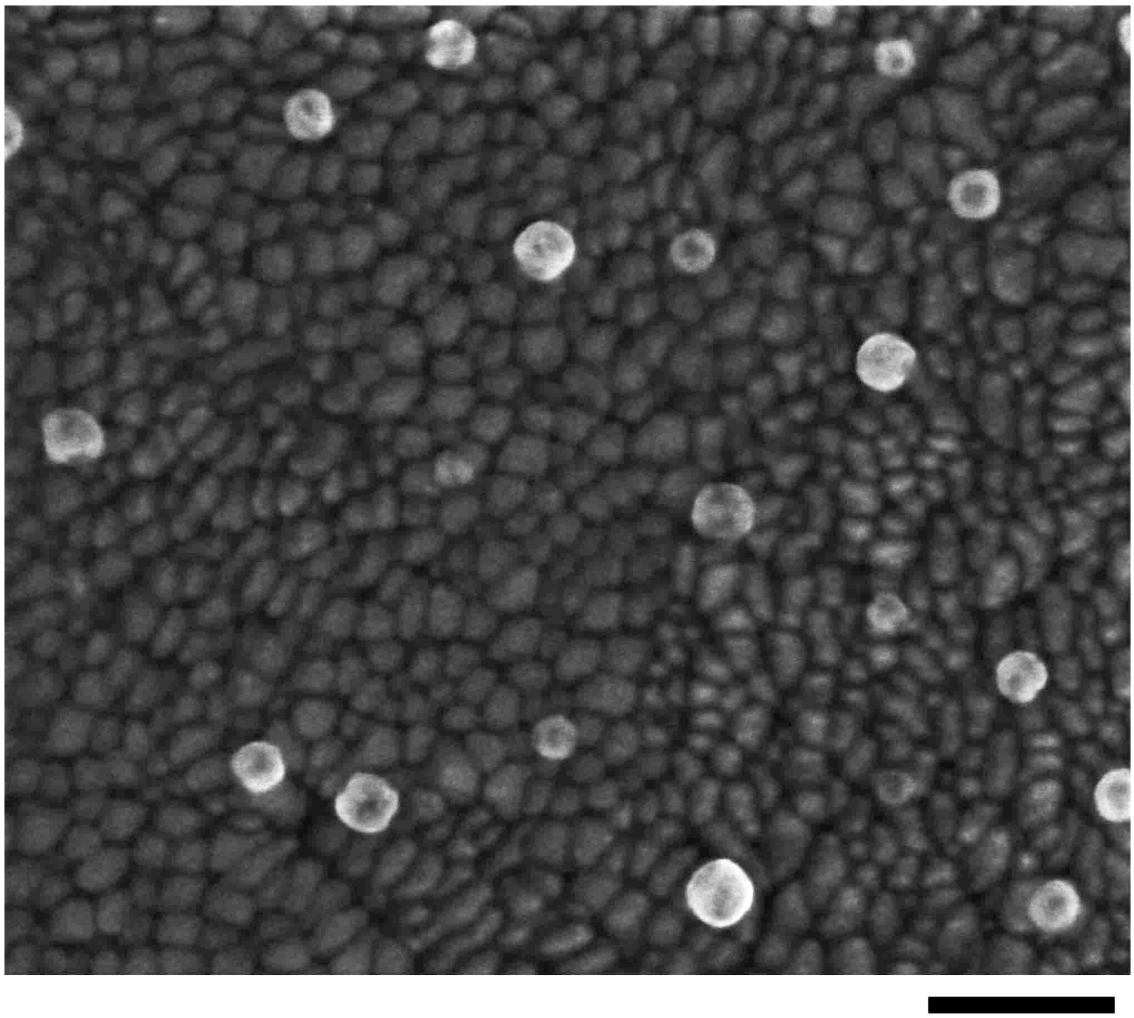


Figure S8. SEM image of avidin-coated vesicles, showing fine structure on their surfaces. This image was taken at acceleration voltage of 2 kV and current intensity of 25 pA and obtained after a few scans (3 minutes in total). Scale bar is 100 nm.

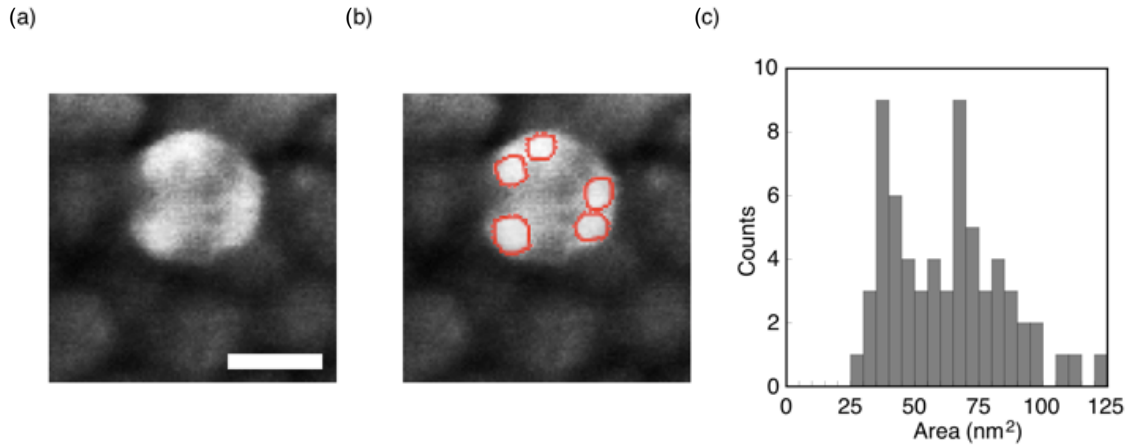


Figure S9. Fine structures on the avidin-coated vesicle. (a) An example image of fine structure taken from Figure S8. Scale bar is 20 nm. (b) A highlighted image of the vesicle in Figure S9a, where the fine structures are enclosed in red. (c) Histogram of the number of fine structures (N = 62) plotted against the area of fine structures.

Comparison of our SEM analysis with conventional SEM methods

There are three key factors to achieve SEM imaging of insulating organic compounds at sub-nm resolution.

(A) Use of ITO as a substrate

The use of conductive ITO substrate for μm-resolution SEM imaging of cultured cells without metal staining was first reported by Fransen.[7] We found that ITO is a good substrate for sub-nm resolution studies. By using the conductive ITO substrate, we avoided metal staining that covers up the details of the structure of the specimen. Si/SiO_2, gold and HOPG substrates could not be used because of the little affinity with the hydrophilic vesicles and proteins (Figure S10). Mica and silica are insulating and hence resulted sample charge-up and no images. The conductive ITO emits more secondary electrons than Si/SiO_2, and contributes to the contrast enhancement in the images.

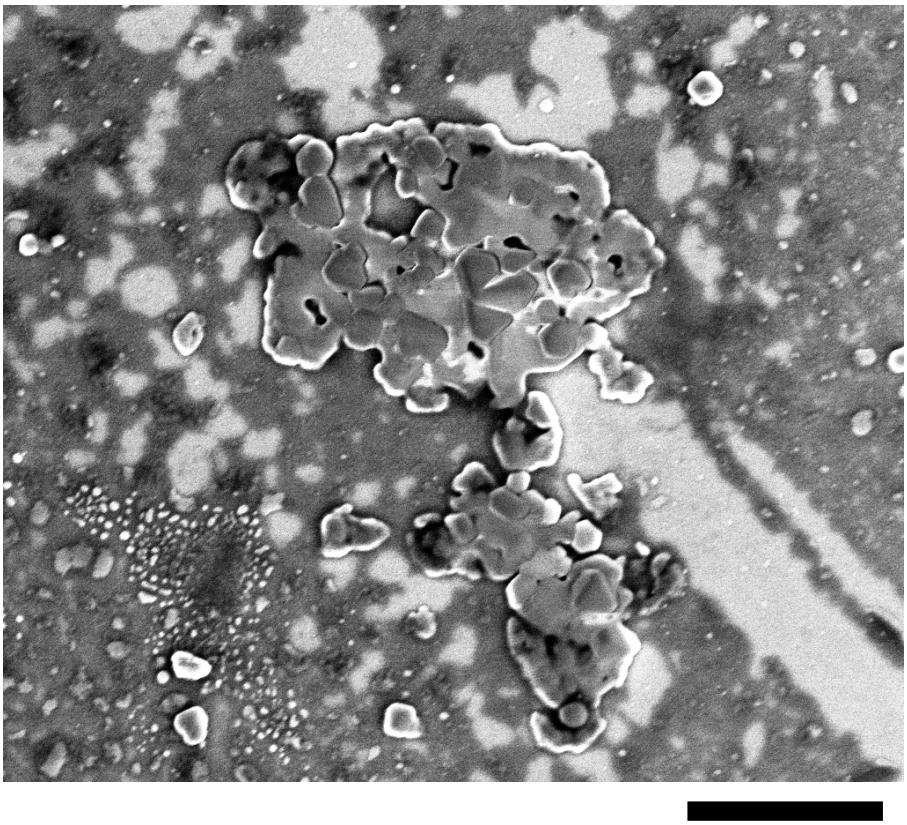


Figure S10. SEM image of a huge aggregate of avidin-coated vesicles on a silicon substrate. Scale bar is 500 nm.

(B) Avoidance of metal-coating

Big problems of SEM imaging of proteins are: (1) Samples must often be left unstained to observe detail of the biological structures; however, lack of staining significantly decreases image contrast. (2) Damage by electrons.[8] Below we show how much the conventional metal coating impedes the image quality of the vesicles on ITO. The 1 nm metal coating in Figure S11b covers the microcrystals of ITO that are clearly visible in Figure S11a. The 5 nm coating completely covers the ITO surface and creates numerous metal particles everywhere on the surface. The average vesicle sizes determined for the intact vesicle, 1-nm metal coating and 5-nm metal coating are 15.5 ± 2.7 nm, 19.7 ± 2.2 nm and 24.6 ± 3.4 nm, respectively (Figure S12). Therefore the ITO substrate is crucial for visualization of intact structure of the vesicle by SEM analysis.

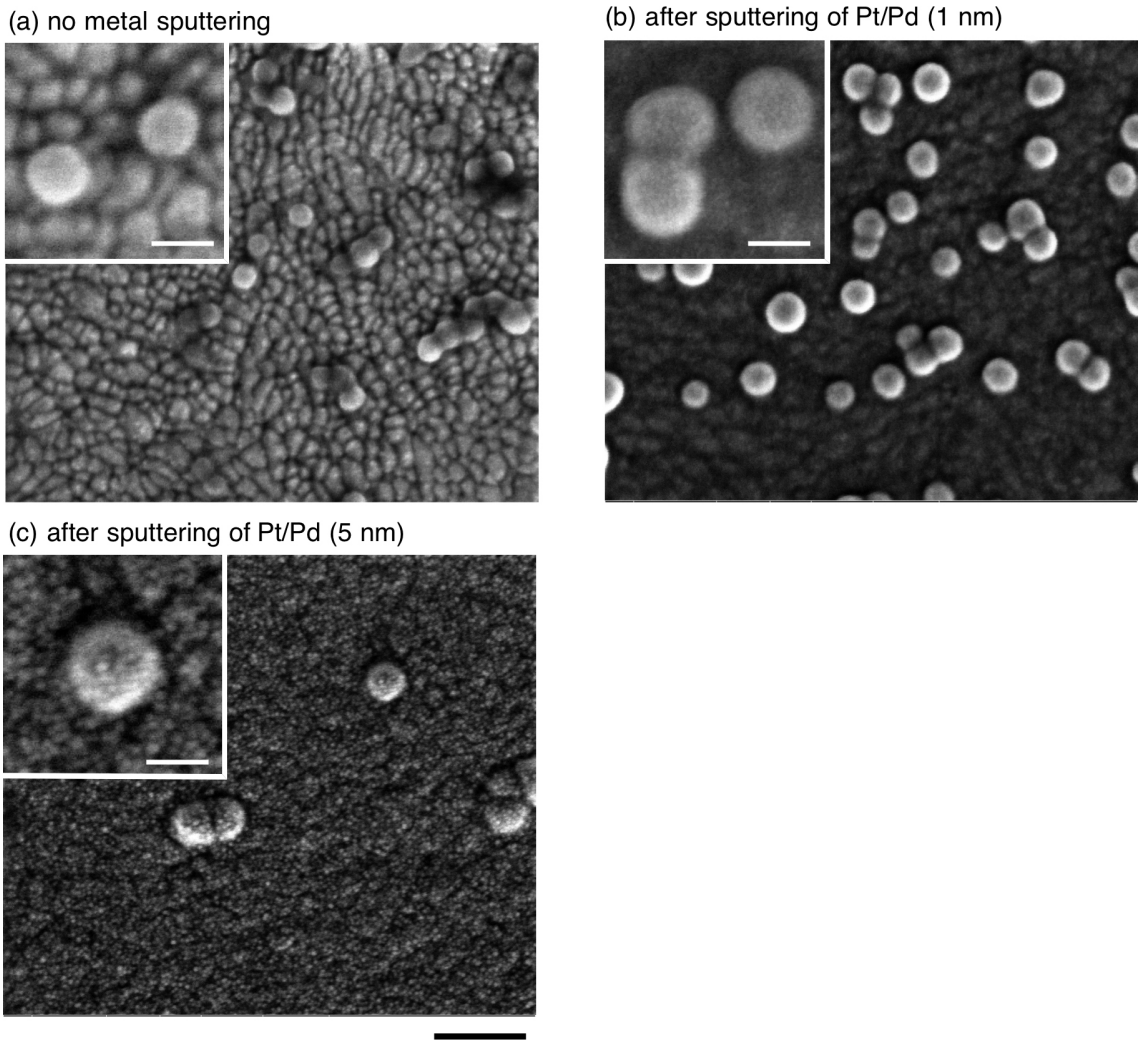


Figure S11. SEM images of avidin-coated fullerene vesicles on glass/ITO substrate. (a) The image of vesicles without metallic coating. (b) The images of vesicles after deposition of Pt/Pd with the thickness of 1 nm. (c) Vesicles after deposition of Pt/Pd with the thickness of 5 nm. Particles of the alloy are clearly observed on the vesicles and ITO grains. Scale bar is 100 nm. Inset: magnified images of the individual vesicles.

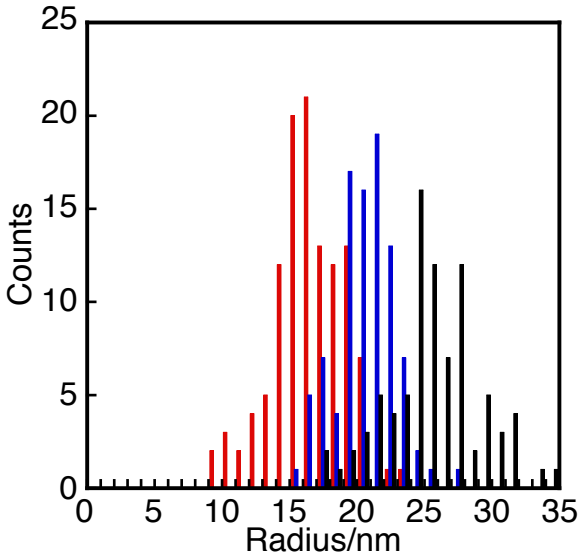


Figure S12. Histogram of the number of the avidin-coated vesicles plotted against the radius of the vesicles in SEM. Red, intact vesicle (N = 116) (Figure S11a); Blue, 1 nm coating of Pt/Pd (N = 93) (Figure S11b); Black, 5 nm coating of Pt/Pd (N = 85) (Figure S11c).

(C) Use of beneficial monochromatic electron beam (unicolor mode) and short working distance (WD)

In addition to the sample preparation, the use of state-of-the-art SEM machine, Magellan 400L by FEI, is the key for high-resolution observation. The instrument features unicolor technology to realize monochromatic electron beam (< 0.2 eV energy spread) and observation at short working distance of 1 mm, which was achieved by a new objective lens technology. Figure S13 shows clear difference of the SEM image quality between the SEM images with the use of unicolor mode and without it.

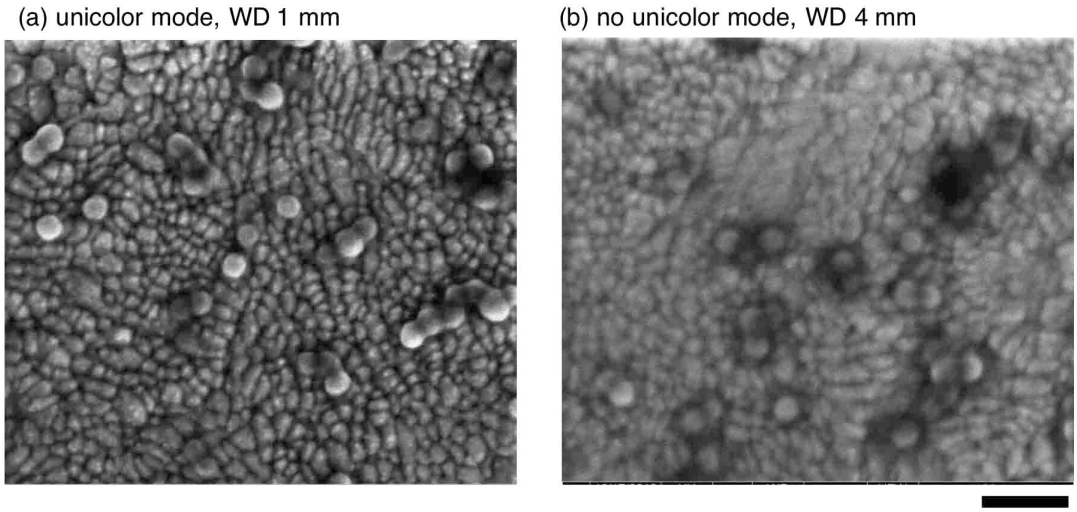


Figure S13. Avidin-coated vesicles on ITO/glass. (a) Image taken under operation in unicolor mode with WD of 1 mm. (b) Without unicolor mode at WD of 4 mm (conventional SEM analysis condition). We could not compare images of the same area by using different mode due to contamination of irradiated area at the first scan of image acquisition. Scale bar is 100 nm.

AFM measurement

AFM measurement was conducted on a Bruker MultiMode 8. Aqueous solution of the **C8(7Y)K** vesicle (2.0 mM, average diameter of 18.4 nm determined by DLS) were deposited on a mica substrate (5×5 mm^2) in aliquot of 2 μL under air. After drying the sample by blowing air for 10 sec and under reduced pressure (5×10^{-2} Pa), the AFM images were obtained by AC mode measurement. The observed width of the vesicles was 20–25 nm, which is 4 to 9 nm larger than that in SEM due to curvature of an AFM tip (ca. 2 nm radius of curvature).

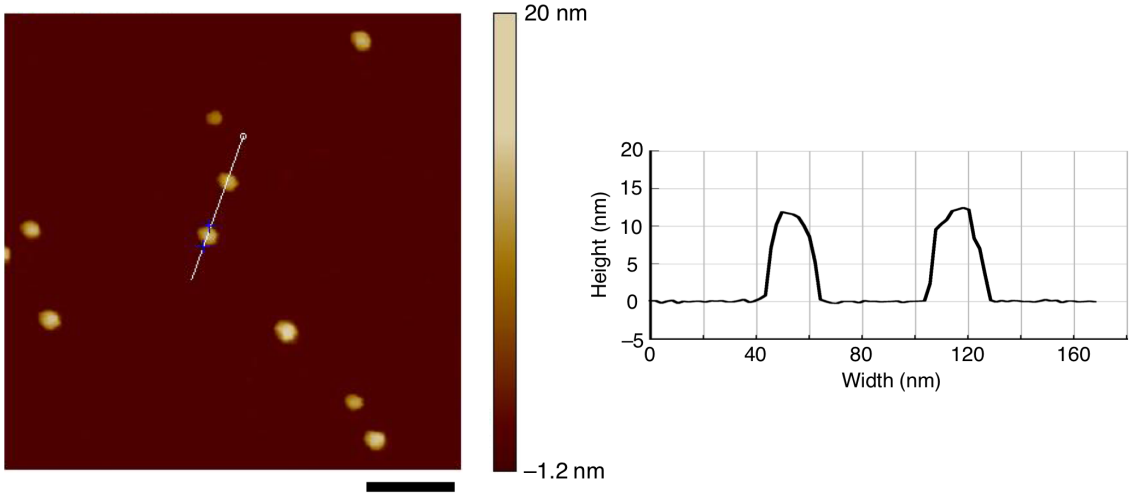


Figure S14. AFM image of **C8(7Y)K** vesicle. Scale bar is 100 nm.

Cytotoxicity assay.

1.0 M of *t*BuOK in THF (70.1 μL, 70.1 μmol) was added to a solution of penta-alkynyl fullerene **C8(7Y)H** (77.0 mg, 46.7 μmol) in THF (3.67 mL) at room temperature. After stirring for 3 h, the portion of the reaction mixture of **C8(7Y)K** (12.5 mM, 0.40 mL, 5.0 μmol) was slowly injected into a solution of doxorubicin (3.07 mg, 5.3 μmol) in distilled-deionized water (2.1 mL) with stirring at 400 rpm for 1 min using a syringe pomp (ISIS CO.) to obtain doxorubicin-encapsulated **C8(7Y)K** vesicle (2.0 mM) in 16% THF/water. THF and water were removed by evaporation at ca. 7 kPa, and the final concentration of **C8(7Y)K** was adjusted to 2.0 mM. Further biotinylation was conducted using this vesicle solution. Stock solutions of **biotin-N_3** (200 mM), copper(II) sulfate (20 mM), (+)-sodium ascorbate (40 mM), and triethylamine (200 mM) in distilled-deionized water was prepared. Doxorubicin-encapsulated **C8(7Y)K** vesicle (500 μL, 1 nmol) was added slowly with stirring to a solution of **biotin-N_3** (25 μL, 0.50 nmol; 5.0 equiv.), copper(II) sulfate (25 mL, 0.50 nmol; 0.50 equiv.), (+)-sodium ascorbate (25 μL, 1.0 nmol; 1.0 equiv.), and triethylamine (25 μL, 5.0 nmol; 5.0 equiv.) at room temperature. After stirring for 6 days, the reaction mixture was passed through a column of Sephadex G-50

fine (GE healthcare Japan, Japan, ϕ 10 × 100 mm) to be separated with unreacted **biotin-N_3** and doxorubicin, which was not encapsulated into the inner aqueous phase of the vesicle, and orange-colored fractions in the eluted solution were collected and diluted with distilled-deionized water to 5.0 mL ([**C8(7Y)K**] = 0.20 mM). The size of biotinylated **C8(7Y)K** vesicle was determined by DLS analysis.

The MTT assay is designed to measure the cell viability. Human liver carcinoma (Hep G2) cells were harvested, collected, and resuspended in 100 μL of D-MEM at a concentration of 2 × 10^4 cells per 1 well over a 96-well plate before the treatment of **C8(7Y)K** vesicles. Biotinylated vesicles and free DOX were diluted to a series of concentrations in 50 μL of PBS to prepare test solutions. Just before addition of the test solutions, the medium was removed and 50 μL of fresh medium was added. The test solutions were added into the wells in the 96-well plate, and the cells were incubated for 48 h at 37 °C in a humidified atmosphere with 5 % of CO_2. At the end of cell culture, the number of viable cells in each wells of the wells was determined by a quantitative colorimetric staining assay using 3-(4,5-di*m*ethyl*t*hiazol-2-yl)-2,5-diphenyl-*t*etrazolium bromide (MTT; Cell Proliferation Kit I (MTT); Roche) as followed the manufactured protocol. Briefly, after incubation for 48 h, 4 μL of MTT labeling reagent was added into the wells in the 96-well plate, and the cells were incubated for 4 h. After incubation, 100 μL of solubilizing solution was added to the wells in 96-well plate, and then incubated overnight at 37 °C in a humidified atmosphere with 5 % of CO_2. Absorbance at 550 nm of each wells was measured, and absorbance at 690 nm was used as a reference wavelength. The results were expressed as the relative value (%) of the control cells, which were incubated parallel with 50 μL of PBS.

The inhibitory concentration (IC_{50}, μM) was defined as a concentration required for inhibiting 50% of the cell growth. Each data point on the curve in Figure15a is indicated as a median value and its error bar based on

three parallel experiments. IC_{50} values and its standard errors were determined by the Dose-Response Logistic Model using software Kaleidagraph;

$$f(x) = \frac{(b-a)}{1+(x/c)^d} = \frac{100}{1+(x/c)^d}$$

where a, b, c, and d indicate minimum value (0%), maximum value (100%), IC_{50} value and gradient of a fitting curve, respectively.

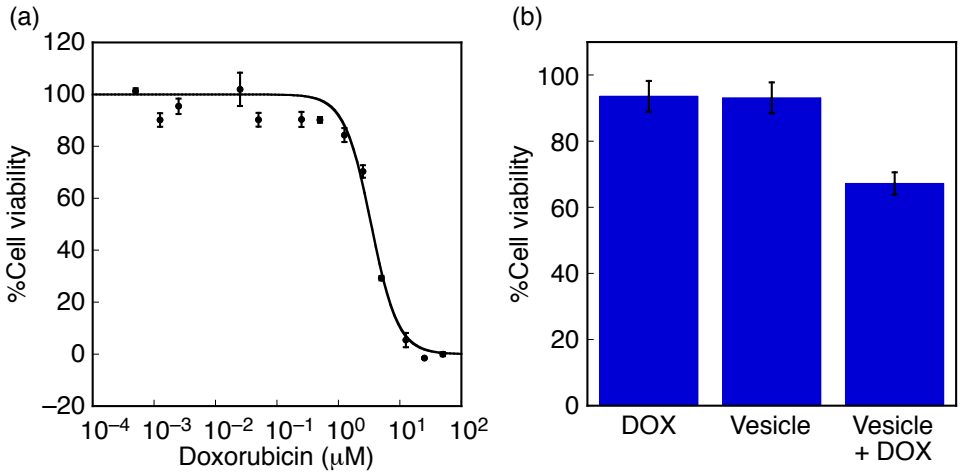


Figure S15. Cell viability assay. (a) Free-doxorubicin. IC_{50} value was 3.2 μM. (b) Cell viability of biotinylated vesicles at 25 nM dose. DOX, free doxorubicin; Vesicle, Biotinylated **C8(7Y)K** vesicle; Vesicle + DOX, doxorubicin-encapsulated vesicle.

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