Electronic Supplementary Information

Reversible conjugation of biomembrane vesicles with magnetic nanoparticles using a self-assembled nanogel interface: Single particle analysis using imaging flow cytometry

Authors

Ryosuke Mizuta[1], Yoshihiro Sasaki[1,*], Kiyofumi Katagiri[2], Shin-ichi Sawada[1], and Kazunari Akiyoshi[1,*]

1. Department of Polymer Chemistry, Graduate School of Engineering, A3-317, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan
2. Department of Applied Chemistry, Graduate School of Engineering, Hiroshima University, 1-4-1 Kagamiyama, Higashi-Hiroshima, 739-8527, Japan

*Address correspondence to:
Department of Polymer Chemistry, Graduate School of Engineering, A3-317, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan.
Email: akiyoshi@bio.polym.kyoto-u.ac.jp
Tel: +81-75-383-2823
Fax: +81-75-383-2590

Experimental Section

Methods

Liposome preparation

DOTAP, DOPS, and NBD-PE were purchased from Avanti Polar Lipids (AL, USA). The DOPC used in this study was COATSOME MC-8181 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Each phospholipid (DOPC, DOTAP, DOPS) and fluorescently labeled lipid (NBD-PE) was dissolved in superdehydrated chloroform (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Liposomes with fluorescently labeled lipid membranes were composed of a molar ratio of DOPC : NBD-PE = 200 : 1. The composition of anionic and cationic liposomes used for ζ potential measurements were DOPC : DOPS : NBD-PE = 180 : 20 : 1 and DOPC : DOTAP : NBD-PE = 180 : 20 : 1, respectively. Each lipid solution was placed in a glass tube and argon gas was introduced to evaporate the solvent. The lipid films were then dried overnight in a vacuum to obtain lipid films. The lipid suspension was obtained by adding 0.1 μm filtered 50 mM HEPES/KOH (pH 7.4) buffer to the thin film and hydrated at a temperature above the phase transition for 4 h, followed by vortex mixing.

The resulting suspension was extruded through a polycarbonate membrane using a mini-extruder.

A thin lipid film consisting of DOPC only was hydrated for 24 h at 37°C in 50 mM HEPES/KOH (pH 7.4) containing calcein at a final concentration of 1 mM. The resulting solution was passed through a polycarbonate membrane using the same technique as for other liposomes. The liposomes with fluorescently labeled inner aqueous phase were then prepared by removing the unincorporated calcein using a PD SpinTrap™ G-25 (Cytiva, Tokyo, Japan).

The lipid content of all liposomes was determined immediately after preparation using the phospholipid C-Test Wako (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Magnetic nanogel preparation

Magnetic nanogels were prepared using a partially modified version of a previously reported method.[37] Specifically, 500 μL of iron oxide nanoparticles (2 mg/mL) dispersed in THF (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) were injected into 10 mL of 0.1 mg/mL rhodamine-modified CHP nanogel dispersion at a rate of 50 μL/min using a syringe pump (Legato110; MUROMACHI KIKAI CO., LTD., Tokyo, Japan). The solution was then lyophilized. The resulting product was dispersed by adding 50 mM HEPES/KOH (pH 7.4) and sonicated at 28 kHz for 5 min in a bath sonicator (VS-100III; AS ONE, Osaka, Japan).

Cell culture

HeLa cells (JCRB9004; JCRB Cell Bank) were cultured in DMEM (Thermo Scientific, MA, USA) containing 10% FBS (Thermo Scientific, MA, USA) and 1% Antibiotic-Antimycotic (AA) (Thermo Scientific, MA, USA). HEK293.2sus cells (ATCC) were cultured in SFM II (Gibco, USA) containing 2% GlutaMAX in a humidified atmosphere with 8% CO2 at 37°C with shaking.

EV isolation

When the density of HEK293.2sus cells reached approximately 1.5~3×10^6/mL, the medium was collected and centrifuged at 4°C and 125 g for 10 min, and then at 4°C and 10,000 rpm for 20 min. The medium was filtered through a 0.22-μm filter (Stericup, Millipore Corp., Bedford, MA, USA). The filtrate was centrifuged at 4°C and 120,000 g for 120 min. The resulting pellet was dispersed in PBS previously passed through a 0.1-μm filter. The suspension was centrifuged again under the same conditions. The resulting pellet was suspended and collected. The protein content of the EVs suspension was determined by BCA assay (Thermo Scientific, MA, USA). The EVs suspension were transferred to 1.5 mL MPC POLYMER COATED TUBE (Sarstedt K.K.) stored at -80°C and thaw before using.

Modification of liposomes with magnetic nanogels

Liposomes fluorescently labeled with NBD and magnetic nanogel were mixed to give final concentrations of 1 mM and 100 μg/mL, respectively. Then, the combined solutions were mixed by inversion using a rotator at room temperature for 24 h. The particle size was determined using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK).

Modification of EVs with magnetic nanogels

HEK293.2sus cells -derived EVs were stained with ExoSparkler EV Membrane Labeling Kit-Green (DOJINDO, KUMAMOTO, Japan). The hybrids of EVs and magnetic nanogels were prepared using a previously reported method.[29] Specifically, magnetic nanogels and EVs dispersed in PBS were mixed so that their final concentrations were 100 μg/mL and 50 μg/mL (protein concentration), respectively, and incubated at 37°C for 24 h.

Imaging flow cytometry setup

All samples analyzed using an AMNIS ImageStreamX Mark II Flow Cytometer (Luminex, TX, USA). Calibration of the various settings of the instrument was performed each time before measuring the samples. The flow rate was calibrated by calculating the sample interval from the average speed of the SpeedBeads (Luminex, TX, USA). The side-scattered light was calibrated by adjusting the laser power at 785 nm so that the intensity when irradiated onto the SpeedBeads was at a default value. Each excitation laser intensities were calibrated by measuring the scattered light intensity when the SpeedBeads were irradiated with each laser.

Single particle analysis of liposome and magnetic nanogel hybrids

100 μL of 1mM Liposomes fluorescently labeled with NBD solution was added to 900 μL of 50 mM HEPES/KOH (pH 7.4), resulting in a 10-fold dilution. This 10-fold dilution was then serially diluted, with 50 μL of sample added to 50 μL of 50 mM HEPES/KOH (pH 7.4). The magnetic nanogel was serially diluted in the same way using 100 μg/mL as the stock solution. As a control, samples of liposomes containing only DOPC and fluorescent-modified liposomes and tritonX-100 (Thermo Scientific, MA, USA) diluted to final concentrations of 1 mM and 0.1%, respectively, were also diluted and measured in the same way. For the hybrids of magnetic nanogel and liposome, the concentration of magnetic nanogel was diluted to 10 μg/mL after mixing for both samples. The laser power was maximized (488 nm : 200 mW, SSC : 70 mW). Signals were detected at Ch1 for brightfield, Ch2 for NBD, Ch3 for rhodamine, and Ch6 for SSC. All measurements were performed at low flow rate and 40× magnification. For the magnetic nanogel-liposome hybrid, the magnetic nanogel was diluted to a concentration of 10 μg/mL and measured in the same way. In order to properly gated, separate and analyze the particles, standard beads (Flow Cytometry Nano Polystyrene and Nano Fluorescent Size Standard Kit, Spherotech, IL, USA) were measured under the above conditions. For the measurement

of standard beads, 5000 particles of each size were measured. The same gating was used for all samples.

To investigate the relationship between the concentration of liposomes introduced and the percentage of hybrids, the liposomes were mixed at 0.005, 0.01, 0.05, 0.1, 1, and 2 mM against 100 μg/mL magnetic nanogel to achieve the hybridization, and single particle analysis was performed.

Single particle analysis of EV and magnetic nanogel hybrids

100 μL of 50 μg/mL (protein concentration) EVs fluorescently labeled was added to 900 μL of PBS, resulting in a 10-fold dilution. This 10-fold dilution was then serially diluted, with 50 μL of sample added to 50 μL of PBS. The magnetic nanogel was serially diluted in the same way using 100 μg/mL as the stock solution. As a control, unstained EVs, EVss and tritonX-100 (Thermo Scientific, MA, USA) diluted to final concentrations of 100 μg/mL and 0.1%, respectively and sample with only staining agent added to buffer were also diluted and measured in the same way. For the hybrids of magnetic nanogel and EV, the concentration of magnetic nanogel was diluted to 10 μg/mL after mixing for both samples. All samples analyzed using an AMNIS ImageStreamX Mark II Flow Cytometer (Luminex, TX, USA). The laser power was maximized (488 nm : 200 mW, 561 nm : 200 mW, SSC : 70 mW). Signals were detected at Ch5 for brightfield, Ch2 for Exosparkler green, Ch3 for rhodamine, and Ch6 for SSC. All measurements were performed at low flow rate and 60× magnification. For the magnetic nanogel-EV hybrid, the magnetic nanogel was diluted to a concentration of 10 μg/mL and measured in the same way. In order to properly gated, separate and analyze the particles, standard beads (Flow Cytometry Nano Polystyrene and Nano Fluorescent Size Standard Kit, Spherotech, IL, USA) were measured under the above conditions. For the measurement of standard beads, 5000 particles of each size were measured. The same gating was used for all samples.

Hybridization of magnetic nanogels and silica particles coated with lipid bilayers

A mixture of 150 μL of 10 mM NBD fluorescently labeled liposomes passed through a 0.05-μm membrane using an extruder and 30 μL of Sicastar (Micromod, Rostock, Germany) with a particle size of 10 μm were inverted and mixed for 2 h. After centrifugation at 2,000 g for 3 min, the supernatant was removed and 200 μL of HEPES buffer was added three times to purify the particles. Magnetic nanogel was mixed with 40 μL of lipid membrane-coated particles to give a final concentration of 100 μg/mL, and the mixture was inverted for 24 h. The washing procedure was performed again, and observation by confocal laser microscopy was performed with an LSM780 (Carl Zeiss, Jena, Germany). Similarly, silica particles, lipid film coated particles, and silica particles mixed with magnetic nanogel were observed. Cross-sectional profiles of the fluorescence images were obtained using ZEN.

Differential scanning calorimetry

GUVs were obtained from a thin lipid film consisting only of DMPC (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) using a static hydration method. Liposomes were then prepared by passing them through a 0.2-μm membrane using an extruder. The prepared liposomes and magnetic nanogel were mixed to a lipid concentration of 10 mM and a nanogel concentration of 500 μg/mL, and the hybrids were prepared by inverting and mixing. 50 μL of liposomes and hybrids with a lipid concentration of 10 mM were placed in a silver pan and measured using a Photo-DSC 204 F1 Phoenix (NETZSCH, Selb, Germany). The temperature was varied at a rate of 5 K/min during the measurement. The analysis was performed on the endothermic peak during the second temperature rise.

Morphological evaluation of magnetic nanogel/liposome hybrids

The prepared hybrids of NBD fluorescently labeled liposomes and magnetic nanogel (nanogel concentration 100 μg/mL) were applied to a 10-μL elastic carbon grid (Okenshoji Co., Ltd, Tokyo, Japan) and allowed to stand for 15 min. The samples were then negatively stained with EM stain (NisshinEM CO., Tokyo, Japan) diluted 5 times with ultrapure water, for 30 min. Observation was performed with an HT7700 transmission electron microscope (Hitachi, Japan). Magnetic nanogels and liposomes were also observed under the same conditions. The acceleration voltage was set at 100 kV.

The hybrids of calcein-loaded liposomes and magnetic nanogels were prepared from liposomes with a lipid concentration of 1 mM and magnetic nanogels with a nanogel concentration of 100 μg/mL. Single particle analysis by imaging flow cytometry was performed on the component particles and hybrids. Calcein solution with the same concentration as 1 mM calcein-encapsulated liposomes was combined with magnetic nanogel and mixed by inversion. The magnetic nanogel was analyzed in the same way. 100 μL of 1mM calcein-loaded liposomes solution was added to 900 μL of 50 mM HEPES/KOH (pH 7.4), resulting in a 10-fold dilution. This 10-fold dilution was then serially diluted, with 50 μL of sample added to 50 μL of 50 mM HEPES/KOH (pH 7.4). The magnetic nanogel was serially diluted in the same way using 100 μg/mL as the stock solution. As a control, samples of liposomes containing only DOPC, fluorescent-modified liposomes and tritonX-100 (Thermo Scientific, MA, USA) diluted to final concentrations of 1 mM and 0.1%, respectively, calcein solution were also diluted and measured in the same way. For the hybrids of magnetic nanogel and liposome, the concentration of magnetic nanogel was diluted to 10 μg/mL after mixing for both samples. All samples analyzed using an AMNIS ImageStreamX Mark II Flow Cytometer (Luminex, TX, USA). The laser power was maximized (488 nm : 200 mW, SSC : 70 mW). Signals were detected at Ch1 for brightfield, Ch2 for NBD, Ch3 for rhodamine, and Ch6 for SSC. All measurements were performed at low flow rate and 40× magnification. For the magnetic nanogel-liposome hybrid, the magnetic nanogel was diluted to a concentration of 10 μg/mL and measured in the same way. The same gating

was used for all samples.

The hybrids were then mixed with tween20 to give a final concentration of 0.5 % and mixed by inverting for 2 h. The solution was magnetically separated, and the fluorescence of the supernatant was measured using a FP-6500 (JASCO, Tokyo, Japan). A solution with a final concentration of 0% tween20 was used in the same way as a control.

Magnetically driven delivery of liposomes

HeLa cells were seeded in 12-well plates at 1×10^5 cells per well, and the hybrids prepared using liposomes fluorescently labeled with NBD and calcein and magnetic nanogel were diluted in serum-free DMEM to give a nanogel concentration of 20 μg/mL and added. A neodymium magnet was placed underneath the 12-well plate and a magnetic field was applied for 1 h. The cells were then washed twice with PBS, stripped using trypsin (Thermo Scientific, MA, USA), and suspended in Stain Buffer (Becton Dickinson, NJ, USA). Measurements were performed using a Cytomics FC500 flow cytometer (Beckman Coulter). The same concentrations of liposomes and hybrids were added in the absence of a magnetic field as a control. The concentration of liposomes was set so that the fluorescence measurement of the solution was equal to the fluorescence intensity of the liposome-derived fluorescence in the hybrids.

Separation of magnetic nanogel/liposome hybrids

The prepared hybrids were mixed with exosome free FBS (final concentration 0%, 10%, 20%, 30%, or 40%) to give a final concentration of 100 μg/mL. The mixture was then incubated at 40°C for 24 h. After magnetic separation using a magnetic stand, the fluorescence of the supernatant was measured. Based on the fluorescence intensity of the solution before magnetic separation, the ratio present in the supernatant to the total liposomes was calculated from the fluorescence of the supernatant. Subsequently, the collected particles were dispersed by magnetic separation, and single particle analysis was carried out. The particles in the combined gating region of magnetic nanogels and hybrids had a count of 10,000 particles.

Separation of magnetic nanogel/EV hybrids

The prepared hybrids were mixed with exosome free FBS so that the final concentration of EV-derived protein was 10 μg/mL. The final concentration of FBS was set at 0%, 10%, 20%, and 30%. After combining, the mixture was incubated at 40°C for 24 h. After magnetic separation using a magnetic stand, the fluorescence of the supernatant was measured. Based on the fluorescence intensity of the solution before magnetic separation, the ratio of EVs present in the supernatant to the total EVs was calculated from the fluorescence of the supernatant.

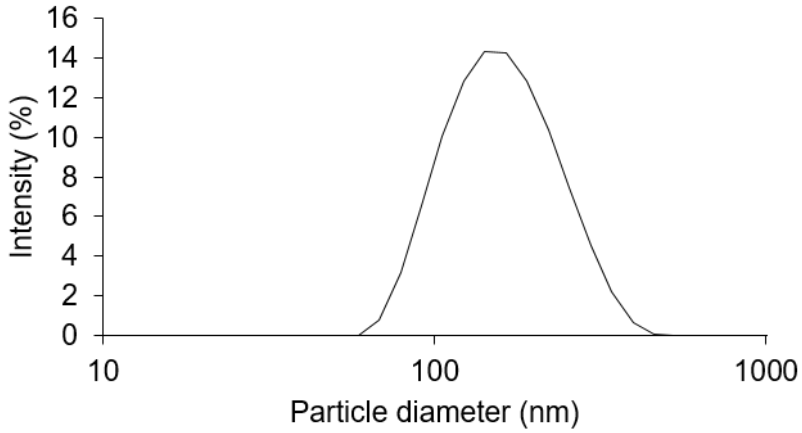

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Figure. S1 Particle size distribution of magnetic nanogels determined by dynamic light scattering.

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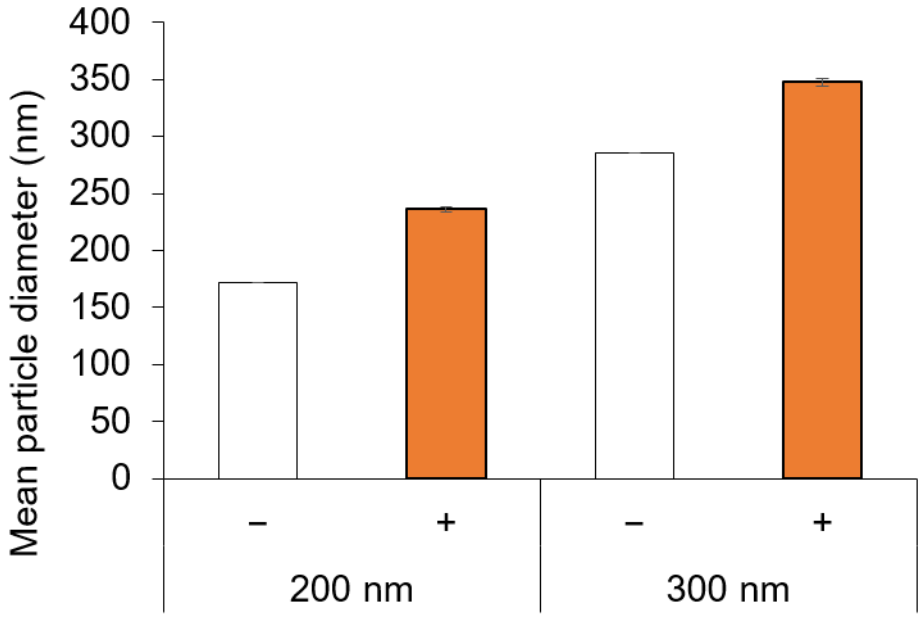

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Figure. S2 Particle size measurements of the magnetic nanogel and liposome hybrids prepared using 0.2 μm and 0.3 μm polycarbonate membranes. + and − denote the presence or absence of complexation with magnetic nanogel, respectively.

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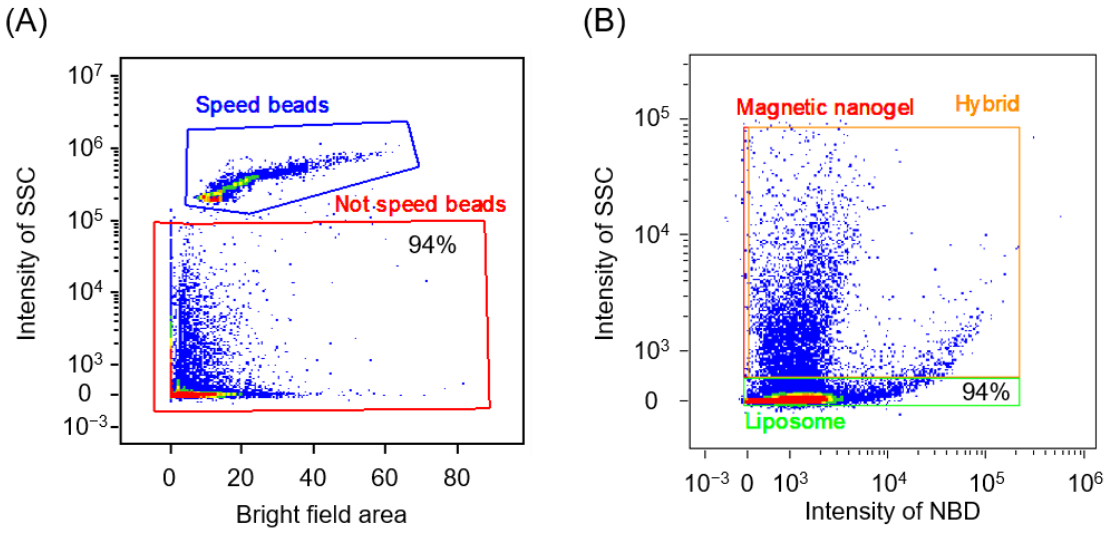

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Figure. S3 Determination of regions for the analysis of magnetic nanogel/liposome hybrids by imaging flow cytometry. Both plots are density plots. (A) Gating to separate speed beads and sample for flow rate control in imaging flow cytometry. (B) Gating to distinguish between magnetic nanogels, liposomes, and hybrids. The boundary line is drawn at the position where more than 90% of the loaded particles can be detected by density plot for magnetic nanogel alone and liposomes alone. The boundary line was drawn at the position where more than 90% of the loaded particles could be detected. The position where almost no single particles appeared was designated hybrid.

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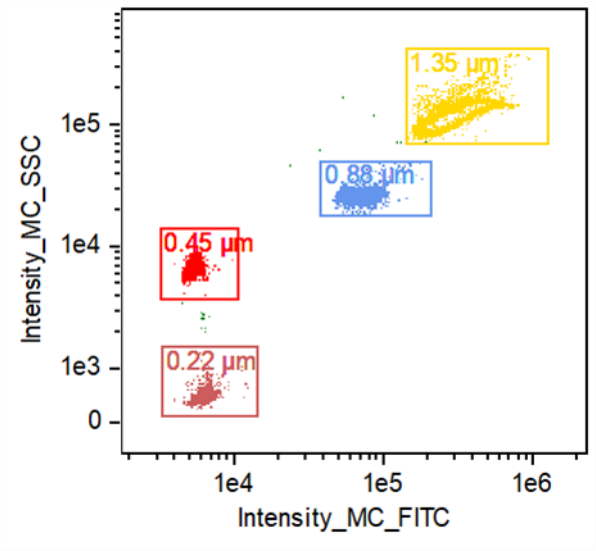

Figure. S4 The result of the measurement of size standard beads using the same settings as for the measurement of liposomes and magnetic nanogels.

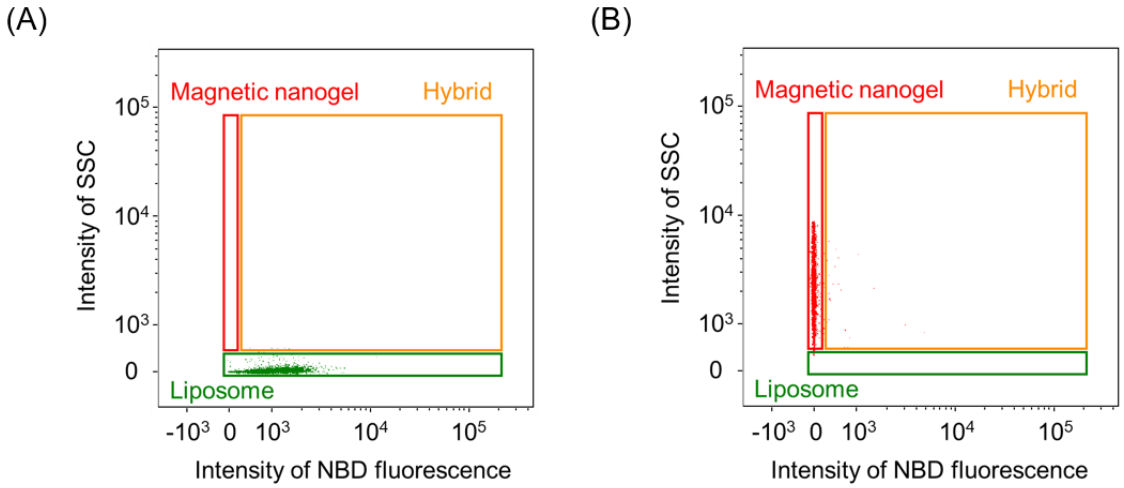

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Figure. S5 Single particle analysis of particles used in the preparation of hybrids. (A) Liposomes fluorescently labeled with NBD in lipid membranes. (B) Rhodamine-labeled magnetic nanogels.

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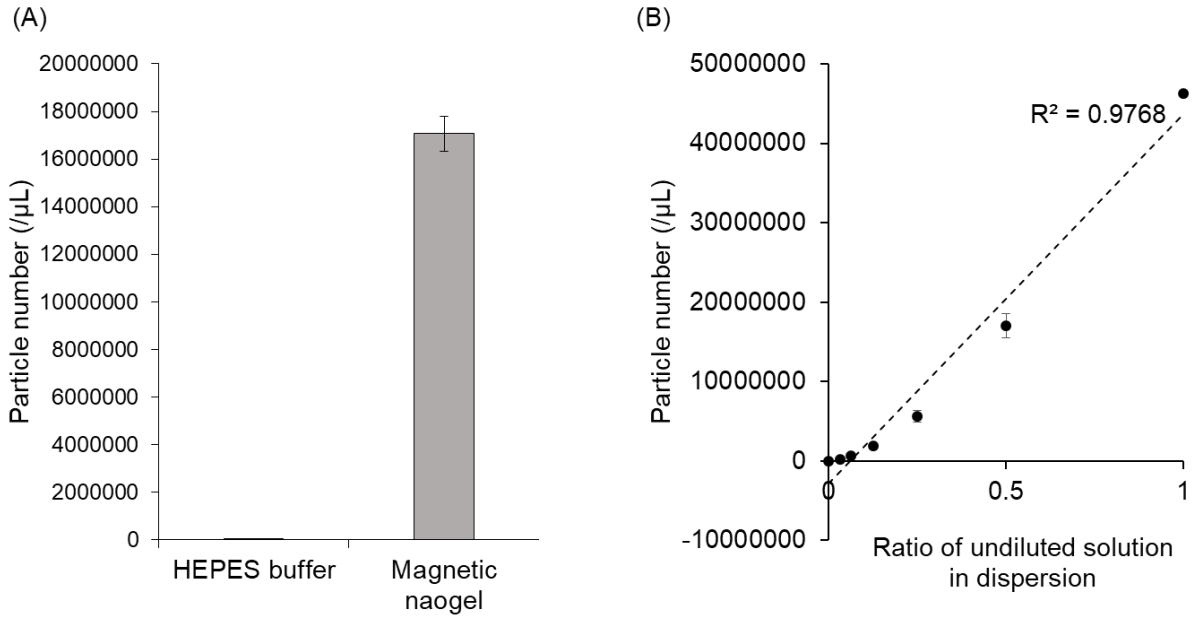

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Figure. S6 Confirmation that magnetic nanogels can be measured in the settings used. (A) Concentration of particles present in the buffer and sample solution used. (B) Serial dilution of the sample solution and the associated change in particle concentration.

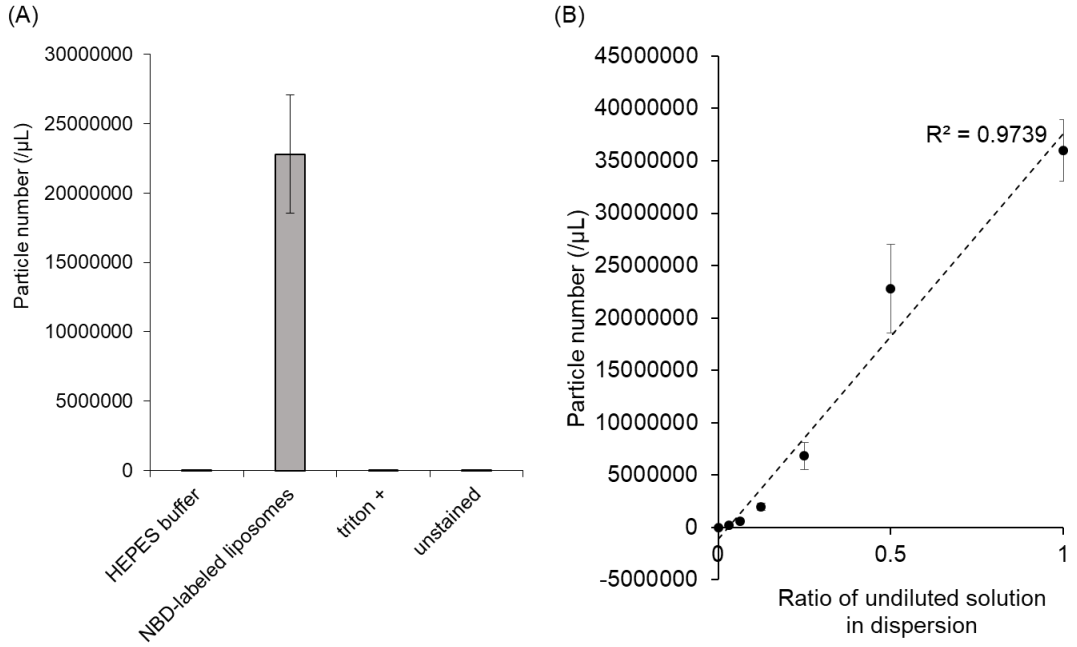

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Figure. S7 Confirmation that liposomes containing NBD-PE in its membrane can be measured in the settings used. (A) The concentration of particles present in the buffer, the various control samples and the sample solution. Triton+ is a sample of the same final concentration as the sample solution exposed to 0.1 % Tritonx-100. Unstained indicates a sample containing the same lipid concentration of liposomes with the same particle size and DOPC-only composition. (B) Serial dilution of the sample solution and the associated change in particle concentration.

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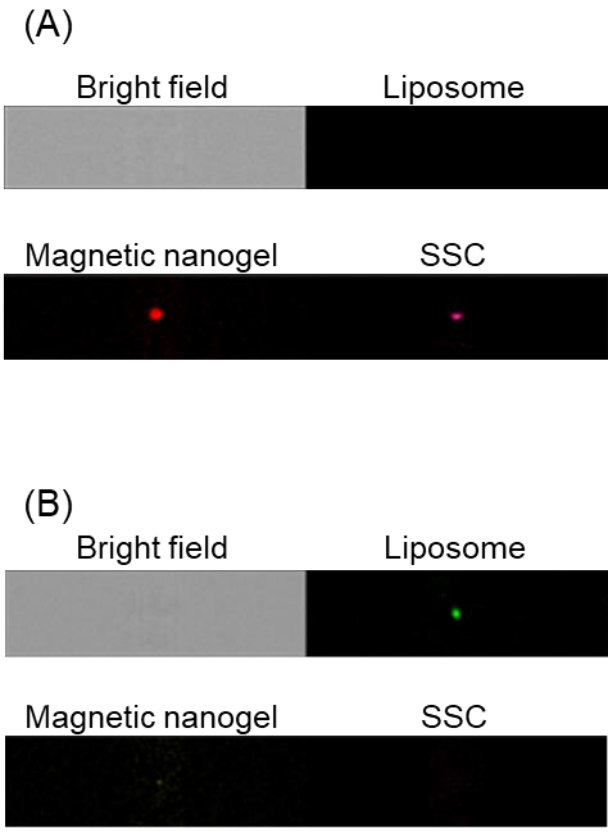

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Figure. S8 Images of nanoparticles obtained by imaging flow cytometry analysis. Liposomes were detected by NBD fluorescence and magnetic nanogels were detected by rhodamine fluorescence. SSC shows the side scattered light. NBD was excited by a 488 nm laser and detected in channel 2. Rhodamine was excited at 488 nm and detected in channel 3. SSC was detected in channel 6. (A) Magnetic nanogel (B) Liposome.

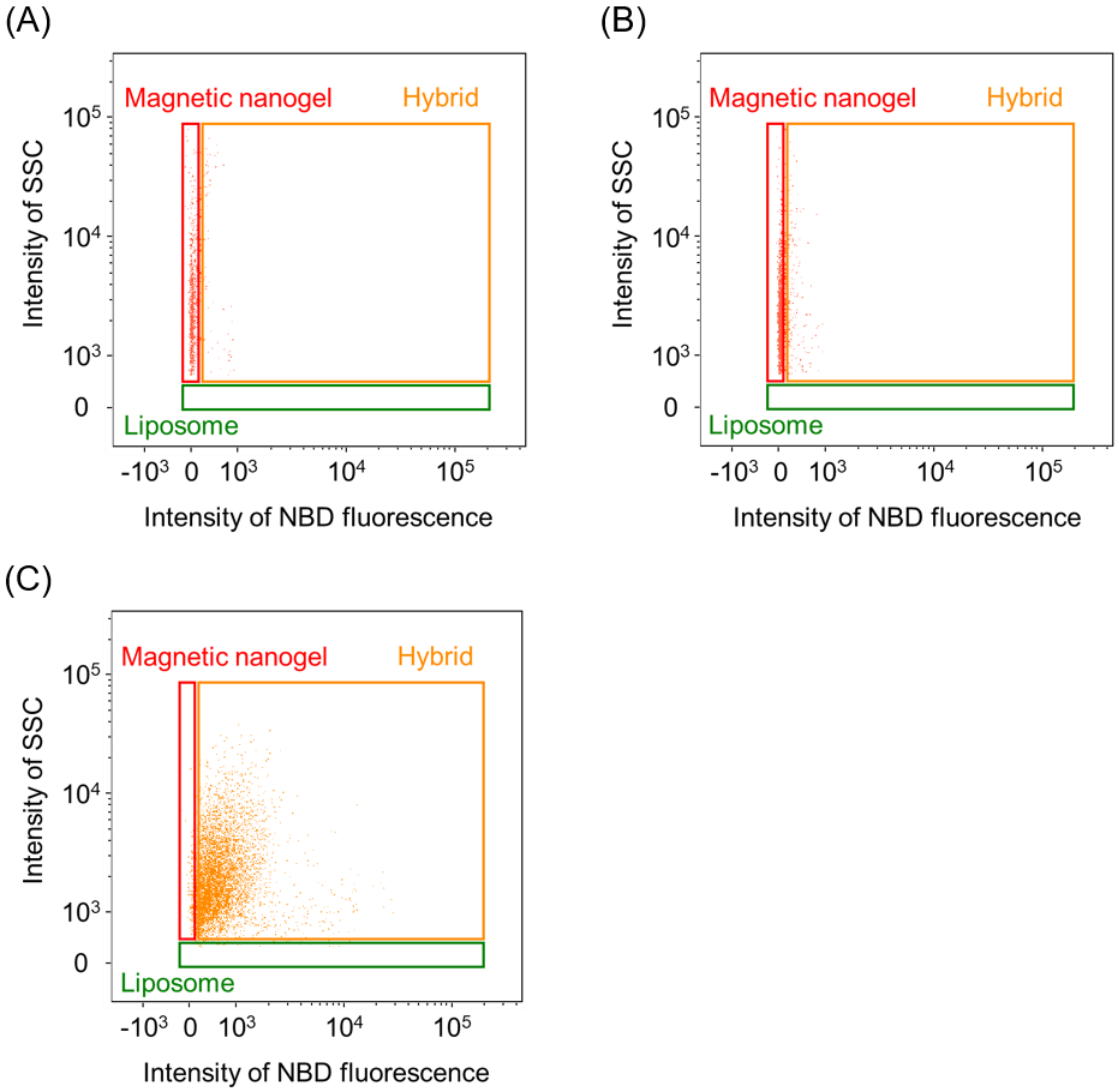

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Figure. S9 Single particle analysis of magnetic nanogel/liposome hybrids using imaging flow cytometry. Distribution of particles in magnetic nanogel and liposome at final concentrations of 100 μg/mL and (A) 0.005 mM, (B) 0.02 mM, and (C) 2 mM.

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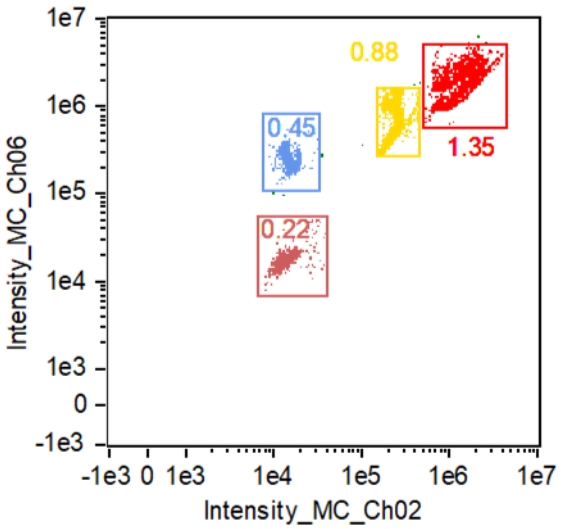

Figure. S10 The result of the measurement of size standard beads using the same settings as for the measurement of EVs and magnetic nanogels.

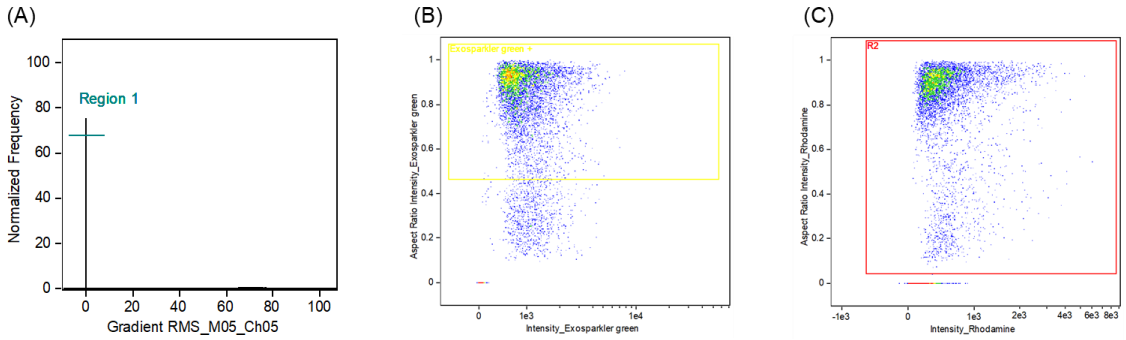

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Figure. S11 Gating used to analyze samples containing EVs. (A) Gating to extract only nanosized particles with no artifacts in bright field. (B) Gating to extract only those particles that show fluorescence of the dye (Exosparkler green) that stained the EV. (C) Gating to extract only magnetic nanogels based on the fluorescence of rhodamine.

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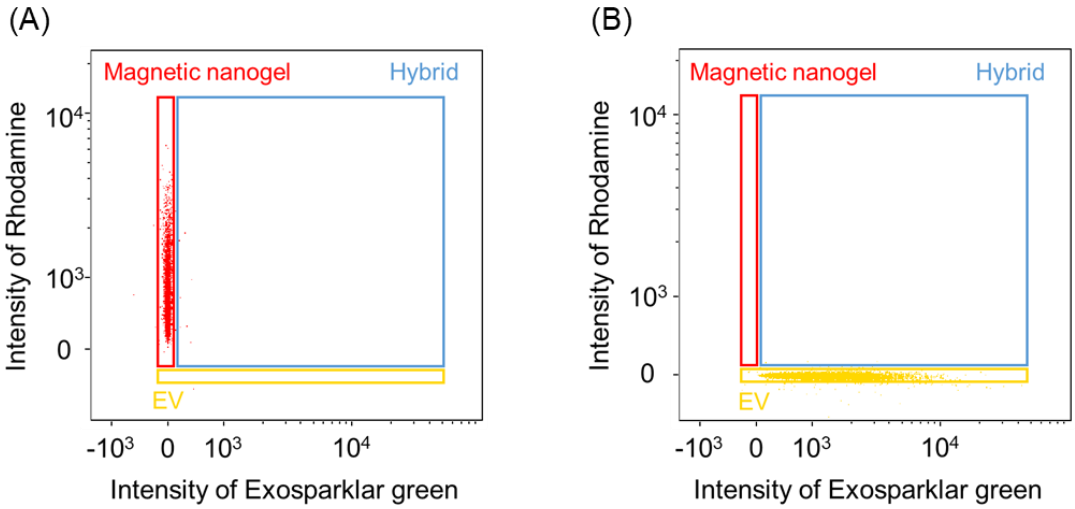

Figure. S12 Single particle analysis of particles used in the preparation of hybrids. (A) Rhodamine-labeled magnetic nanogels. (B) HEK293 derived EVs fluorescently labeled with Exosparkler green.

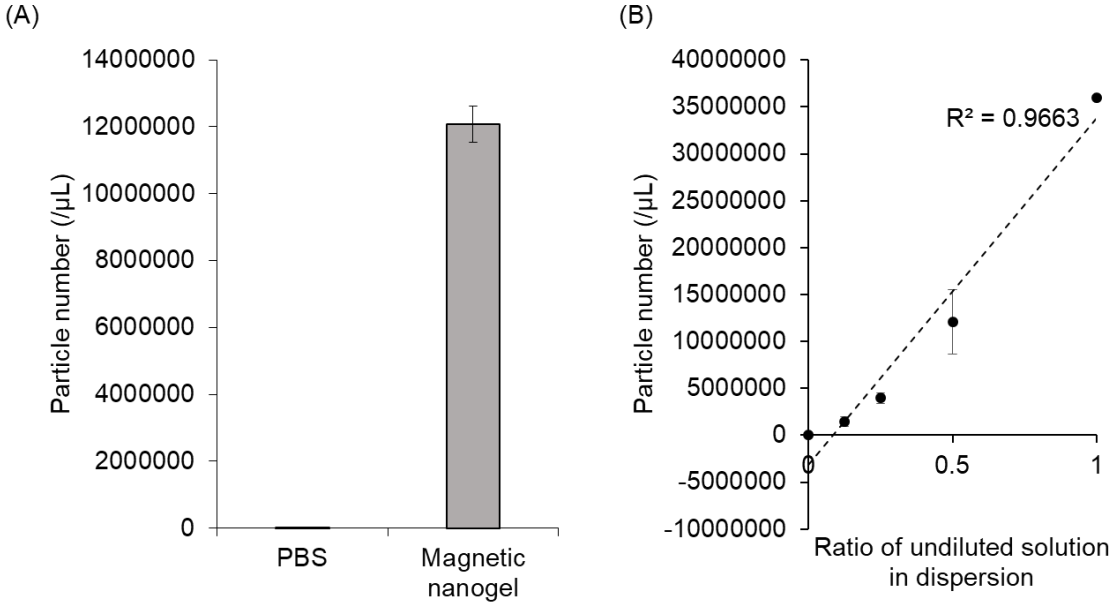

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Figure. S13 Confirmation that magnetic nanogels can be measured in the settings used. (A) Concentration of particles present in the buffer and sample solution used. (B) Serial dilution of the sample solution and the associated change in particle concentration.

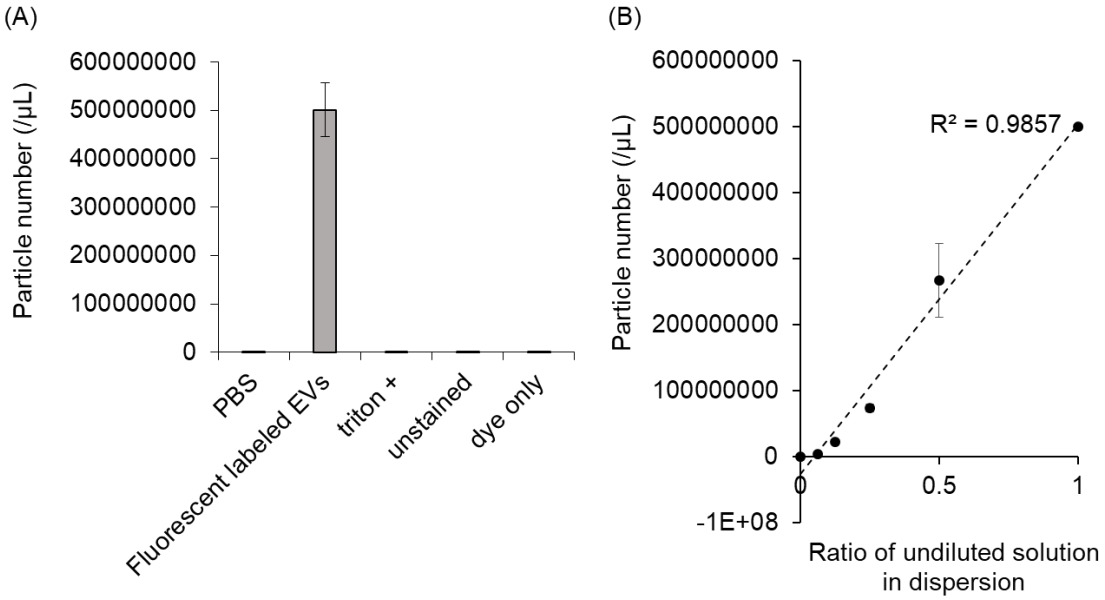

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Figure. S14 Confirmation that EVs fluorescently labeled with Exosparkler green can be measured in the settings used. (A) The concentration of particles present in the buffer, the various control samples and the sample solution. Triton+ is a sample of the same final concentration as the sample solution exposed to 0.1 % Tritonx-100. Unstained indicates a sample containing the same protein concentration of unstained EVs. Dye only is a sample in which only the stain used to label EV is dispersed in the buffer. (B) Serial dilution of the sample solution and the associated change in particle concentration.

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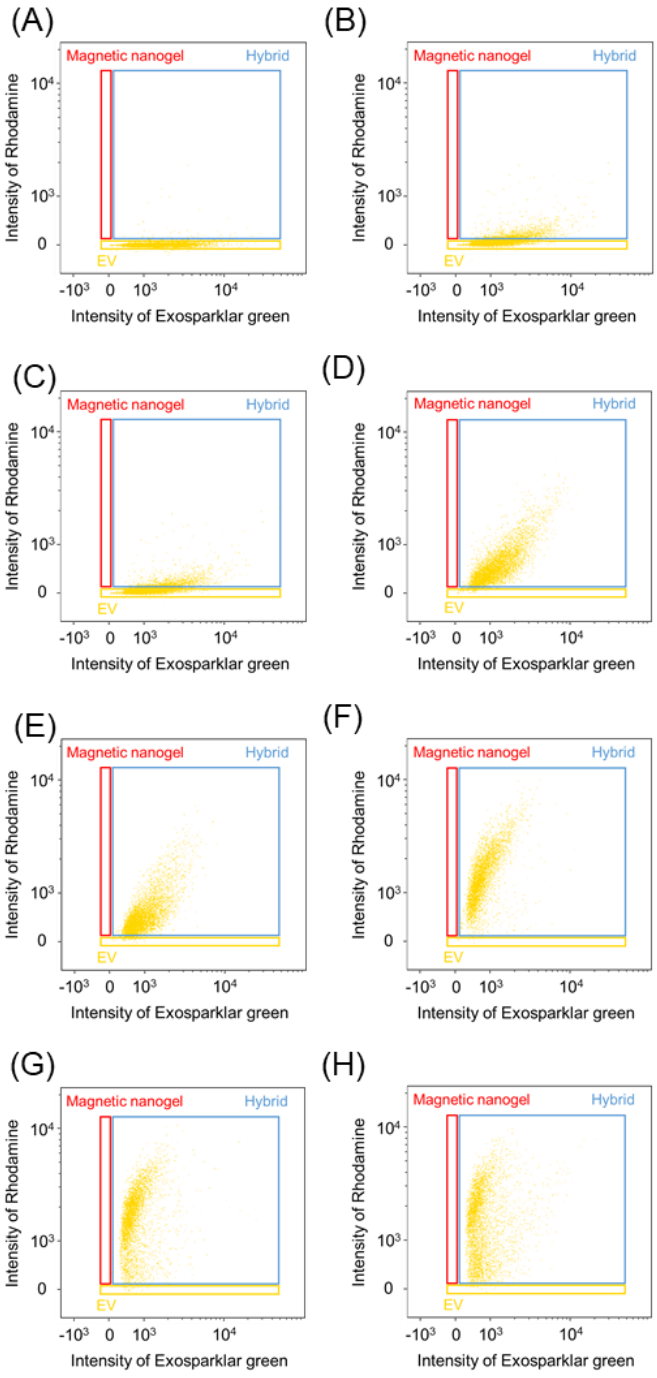

Figure. S15 Single particle analysis of magnetic nanogel/EV hybrids using imaging flow cytometry. Distribution changes of particles detected as EVs when magnetic nanogels of (A) 1, (B) 5, (C) 10, (D) 20, (E) 50, (F) 250, (G) 500, and (H) 1000 μg/mL were mixed with EVs of 50 μg/mL final protein concentration.

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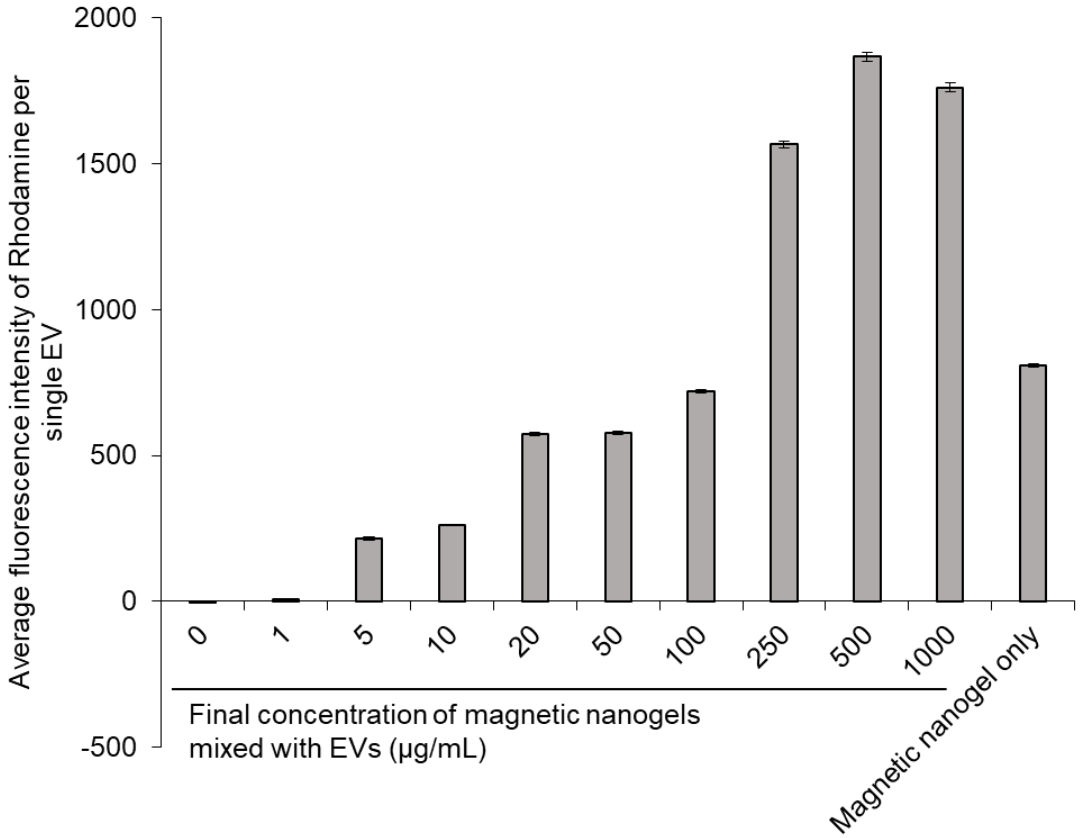

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Figure. S16 Average fluorescence intensity of nanogel-derived rhodamine per single extracellular vesicle mixed with various concentrations of magnetic nanogel. The average fluorescence intensity per single particle of magnetic nanogel is also shown for reference. All values were obtained by imaging flow cytometry.

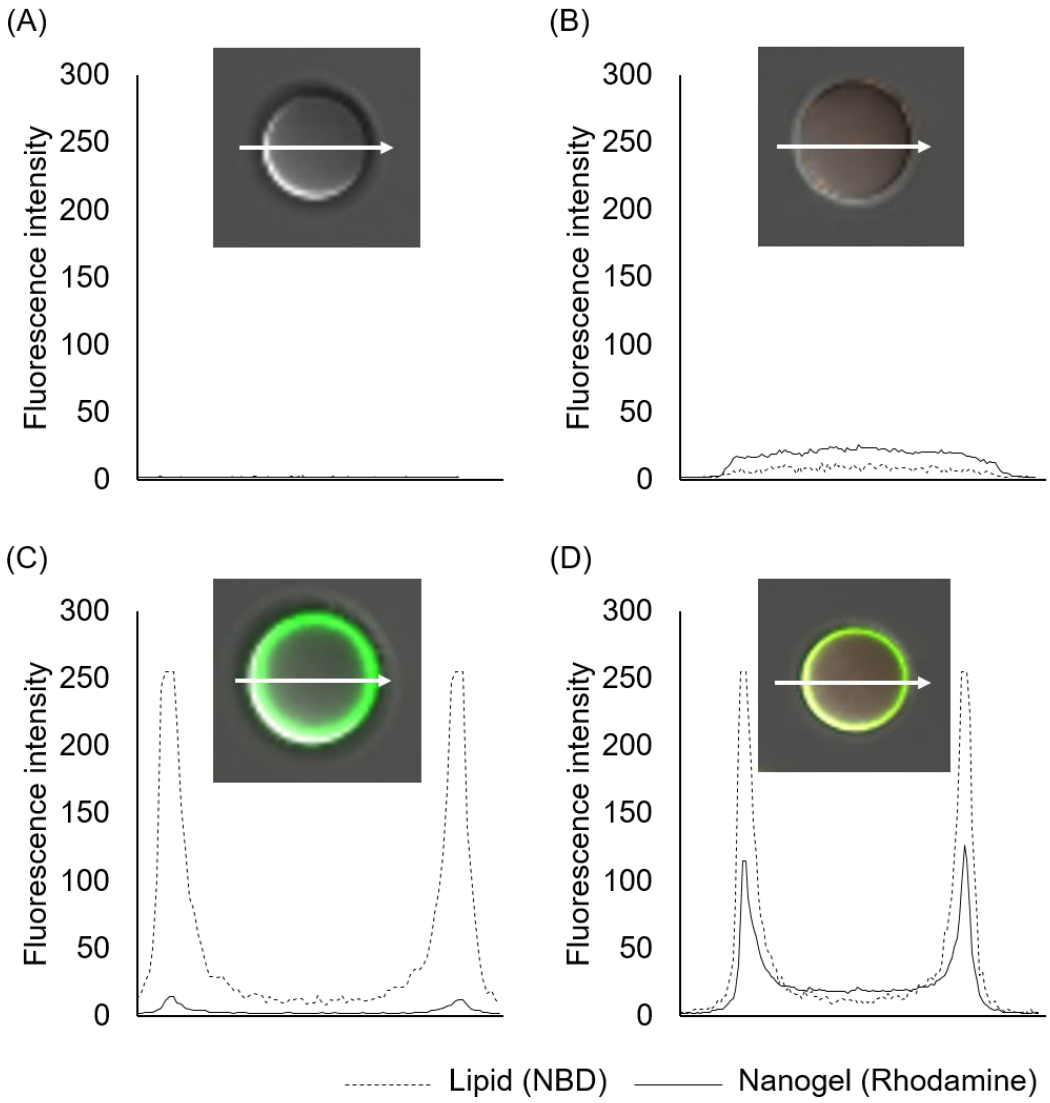

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Figure. S17 Cross-sectional fluorescence profiles of representative particles in confocal laser microscopy of silica particles coated with lipid bilayers hybridized with magnetic nanogels. The dashed line represents the fluorescence of the lipid membrane, and the solid line represents the fluorescence from the magnetic nanogels. (A) silica particles. (B) A mixture of silica particles and magnetic nanogels. (C) Silica particles coated with lipid bilayers. (D) A mixture of silica particles coated with lipid bilayers and magnetic nanogels.

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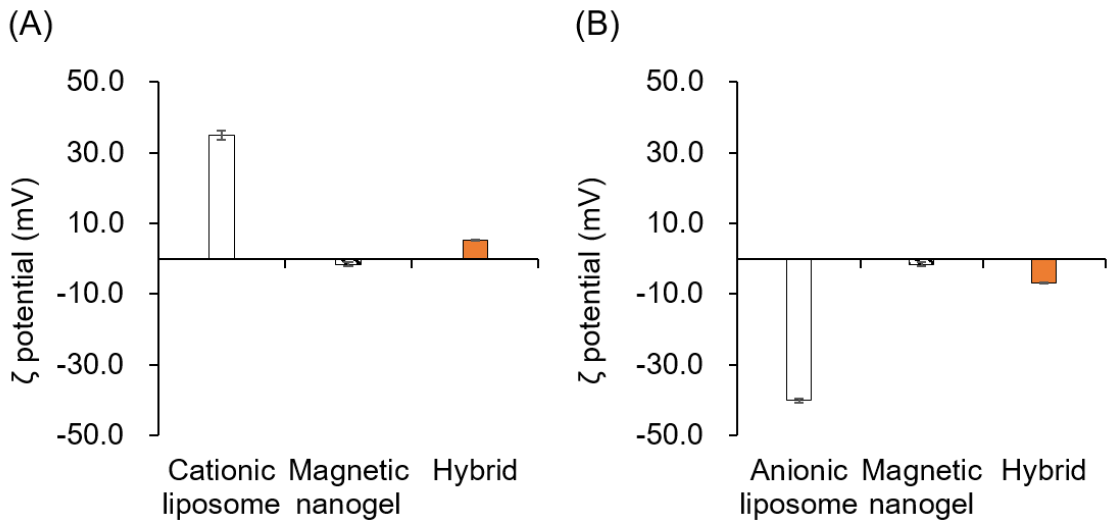

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Figure. S18 Hybridization of different types of liposomes with magnetic nanogels. For each experiment, three samples were measured and their means and standard deviations were calculated. (A) Change in ζ potential of cationic liposomes hybridized with magnetic nanogels. (B) Change in ζ potential of anionic liposomes hybridized with magnetic nanogels.

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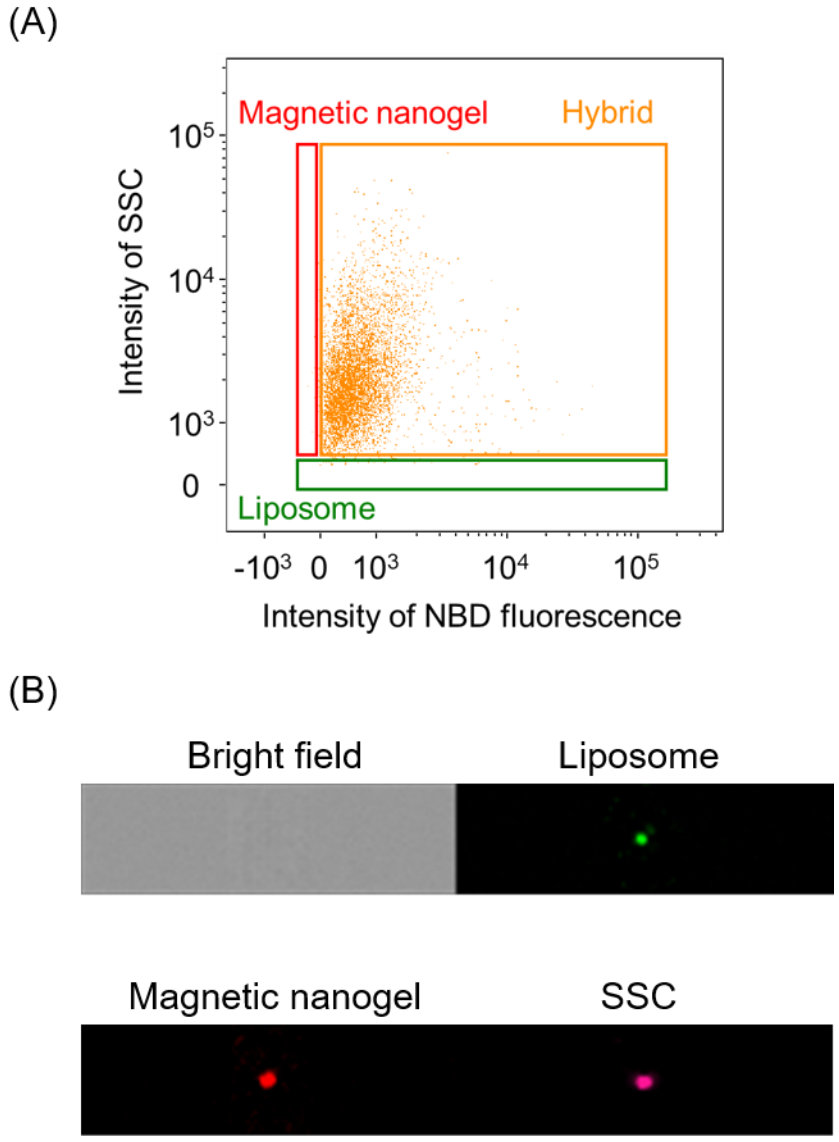

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Figure. S19 Single particle analysis of hybrids composed of magnetic nanogel and cationic liposomes using imaging flow cytometry. (A) Distribution of particles in magnetic nanogel and liposome at final concentrations of 100 μg/mL and 1 mM, respectively. (B) Fluorescence image of particles detected in the region of the hybrid.

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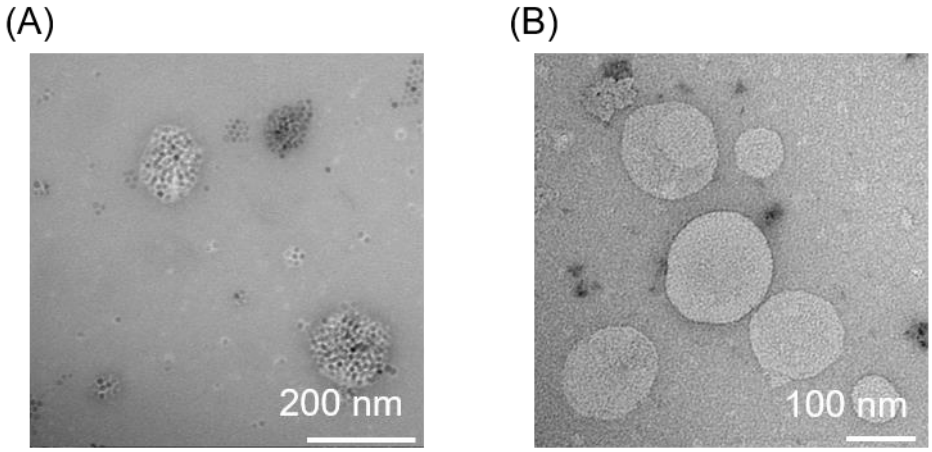

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Figure. S20 TEM images of the particles used in hybrid preparation. For observation, negative staining was performed using EM stain diluted three times with ultrapure water. (A) Liposomes. (B) Magnetic nanogels.

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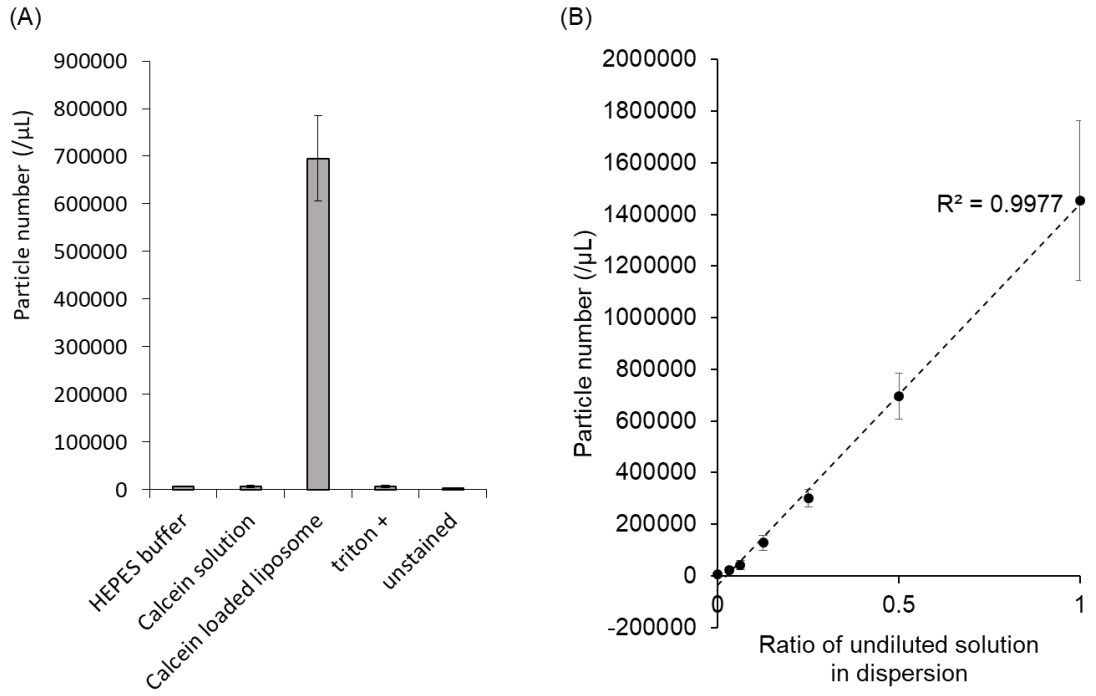

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Figure. S21 Confirmation that calcein-loaded liposomes can be measured in the settings used. (A) The concentration of particles present in the buffer, the various control samples and the sample solution. Calcein solution represents the calcein dissolved in buffer so that the fluorescence intensity is comparable to that of the liposome solution. Triton+ is a sample of the same final concentration as the sample solution exposed to 0.1 % Tritonx-100. Unstained indicates a sample containing the same lipid concentration of liposomes with the same particle size and DOPC-only composition. (B) Serial dilution of the sample solution and the associated change in particle concentration.

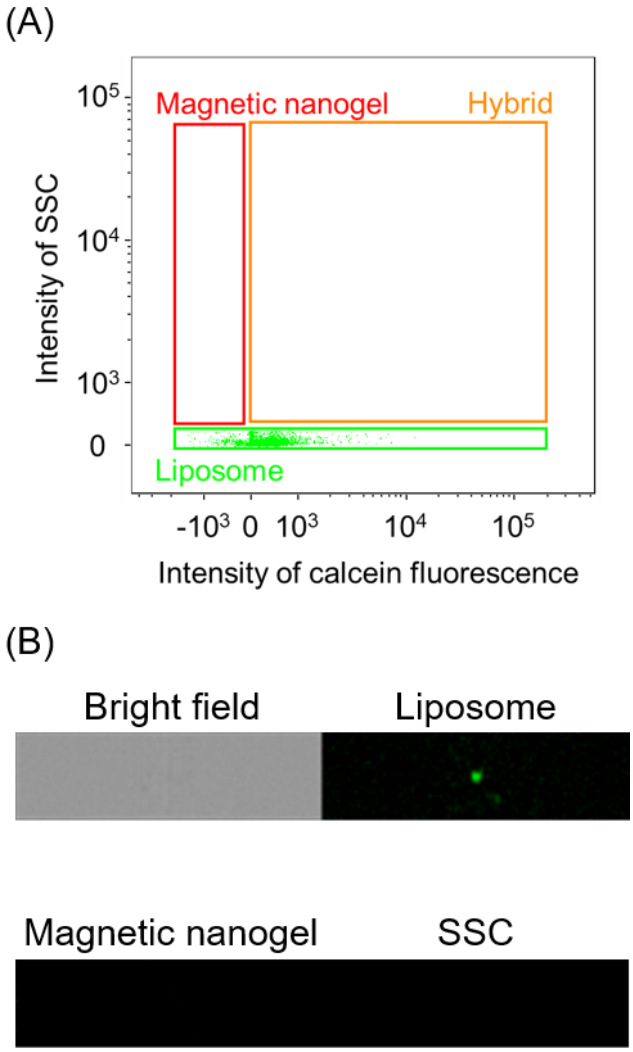

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Figure. S22 Single particle analysis of calcein loaded liposomes using imaging flow cytometry. (A) Distribution of detected particles. (B) Fluorescence image of particles detected in the region of the liposome.

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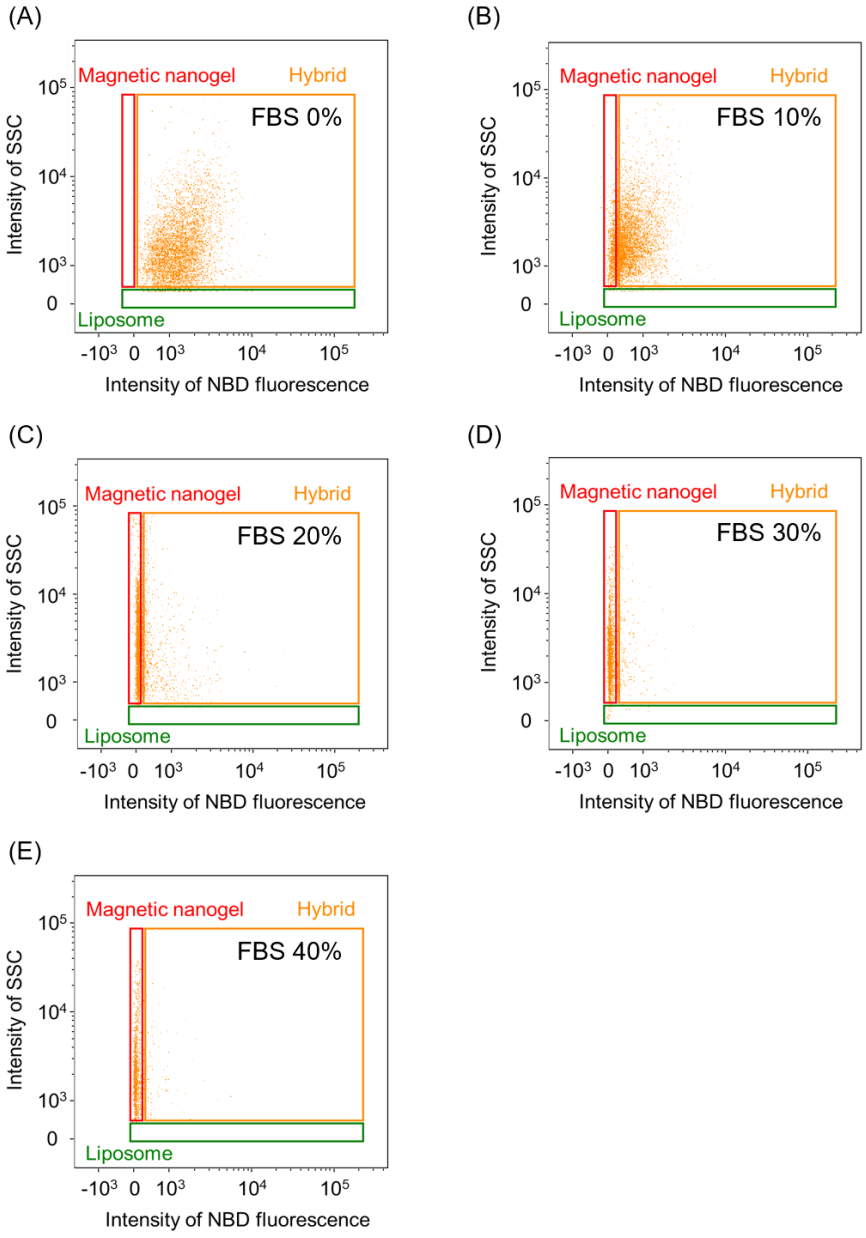

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Figure. S23 Single particle profiles by imaging flow cytometry of magnetic nanogel/liposome hybrids exposed to various protein concentration conditions. Final FBS concentrations of (A) 0%, (B) 10%, (C) 20%, (D) 30%, and (E) 40%.