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Supplementary Information

Highly stable lipid-encapsulation of fluorescent nanodiamonds for bioimaging applications

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1. Experimental:

Synthesis of FND-PCL. 100-nm-sized fluorescent nanodiamonds (FNDs) were produced by He^+ ion irradiation to diamond powders containing ~100 ppm of atomically isolated nitrogen with a irradiation energy of 40-keV, followed by annealing at 800 °C for 2 h under reduced pressure, air oxidation at 450 °C for 2 h, as previously described.[1] The hydrodynamic size determined by dynamic light scattering (DLS) was 100 ± 20 nm. 2.0 mg of diyne PC [DC(8,9)PC] (Avanti Polar Lipids) and 85 μg of Cholesterol (Sigma) were dissolved in 2 ml of THF and added to an 10 mL of deionized distilled water (DDW) containing 1 mg of FNDs. The mixture was sonicated for 10 min, and then THF was removed by rotary evaporation, yielding lipid-coated FNDs. Cholesterol served as a stabilizer for lipid-coated FND. The solution of lipid-coated FNDs was UV-irradiated for 90 minutes at a wave length of 254 nm (UVS-28, UVP) in order to polymerize diyne PC, yielding photo-crosslinked lipid-coated FND (FND-PCL). The FND-PCL were centrifuged (10,000 *g*, 5 min) and washed three times with DDW, and filtered using 0.22 μm Millipore nylon filter (Merck Millipore).

Synthesis of FND-PCLCOOH and FND-PCL-anti-CD44. 2.0 mg of diyne PC [DC(8,9)PC], 7.6 μg of 10,12-Tricosadiynoic acid (TCI) and 85 μg of Cholesterol were dissolved in 2 ml of THF and added to 10 mL of DDW containing 1 mg of FNDs. Following processes of the reaction, UV irradiation, and centrifuge/wash processes are similar to that of FND-PCL. After the processes, we obtained COOH functionalized FND-PCL (FND-PCLCOOH). 100 μg of FND-PCLCOOH in 1 ml of DDW were reacted with 720 μg of N-Hydroxysuccinimide (NHS) and 600 μg of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) for 30 min at room temperature to activate the COOH groups. We then centrifuged the solution and wash 1 time by DDW, and the pellet was suspended in 1 ml of PBS. 10 μl of anti-CD44 antibody (purified mouse IgG1, BioLegend) was added to the solution and gentle shaking for 1 h at room temperature, then the solution was washed PBS by two times, yielding FND-PCL-anti-CD44.

Characterization of the FND samples. UV-vis measurements were carried out on an U-3310 (HITACHI) operating at a resolution of 1 nm in the region of 200-800 nm. FNDs were dispersed in DDW at a concentration of 10 μg/mL, and measured UV-vis absorption spectrum after the various irradiation time of UV at 254 nm. Fourier transform infrared spectroscopy (FTIR) spectra were obtained on a Bruker VERTEX 70 using standard KBr-pellets in the range of 4000-800 cm^{-1} with a resolution of 4 cm^{-1}. The average size of FNDs was analyzed by DLS and zeta potential were measured using a Delsa Nano C (BECKMAN COULTER). We note that size distributions are represented by the particle number. Fluorescence spectra were recorded using a spectrometer (C7473 Hamamatsu). Solution of FNDs in DDW (100 μg/ml) was illuminated using a 532 nm laser at 20 mW.

Cell culture and nonspecific adsorption assessment of FNDs on live and fixed HeLa cells. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) that was supplemented with 10% fetal bovine serum and 1% penicillin, and incubated in 5% CO_2 incubator at 37 ^{o}C. Cell fixation was performed using 4% paraformaldehyde (Electron Microscopy Sciences) for 10 minutes, and then repeatedly washed with PBS. After the washing, fixed cells were treated with 3% BSA solution in PBS for blocking. HeLa cells were plated at a density of 5×10^5 cells per 35 mm dish in DMEM and cultured for 22-26 h in incubator. After incubation, culture medium was removed and subsequently FND-containing medium (0, 10, 20, 50, or 100 µg/ml) was added to the cell culture dish. After incubation for one hour, cells were washed with PBS extensively. After cells were harvested by trypsin treatment or cell scraper, they were analyzed by flow cytometry (FACSArray Bioanalyzer, BD Biosciences). This experiment was also performed to fixed cells.

Cell viability assay. Dehydrogenase activity detection in viable cells was measured with the colorimetric assay cell counting kit-8 (CCK-8, Dojindo). HeLa cells (5000 cells in 100 µL culture medium) were seeded in 96-wells plates for 22-26 h. FND samples were then added to cells, and cells were incubated for 22-26 h at 37 ^{o}C. The absorbance was measured at 450 nm by using a plate reader (Multiskan, Thermo) 1 h after the addition of CCK-8.

Specific targeting to CD44 on HeLa cells membrane. HeLa cells were plated at a density of 5×10^5 cells per 35 mm dish cultured for 22-26 h. After incubation, DMEM was removed and subsequently FND-PCLCOOH, FND-PCL-anti-CD44, and isotype control antibody modified FND (FND-PCL-isotype) in DMEM (100 µg/ml) was added to the cell culture dish. Regarding pretreatment control experiments, cells were pretreated with free anti-CD44 antibody (25 µg/ml) for one hour in order to saturate the antibody-binding sites before the treatment with FND-PCL-anti-CD44. After incubation for one hour, cells were extensively washed with PBS, subsequently analyzed by flow cytometry and confocal microscopy. Regarding the experiment of confocal microscopy, cells were treated with Hoechst 33342 to stain their nuclei. Hoechst 33342 and FND were excited by 405 and 532 nm laser, respectively, and fluorescence was collected through an oil-immersion objective (63×, NA 1.4) and detected using a photomultiplier and hybrid detector, respectively.

CLEM imaging

HeLa cells (1 x 10^5) were seeded on 35 mm dishes with gridded glass bottoms (MatTek) one day before experiment. CD44 antigens on HeLa cell surface were targeted as described in the specific targeting section. The samples were then dehydrated through a serial concentration of EtOH/DDW (30%, 50%, 70%, 90%, 95% and 100%) for 15 min in each step and fully dried by critical point drier

(Samdri PVT-3B, Tousimis). The fluorescence images were collected by the confocal microscope and the EM images were taken by table-top SEM (Phenom ProX) for the same cells. The fluorescence images were then superimposed on the EM images after a bit enlargement and appropriate angle tilt.

2. Figures:

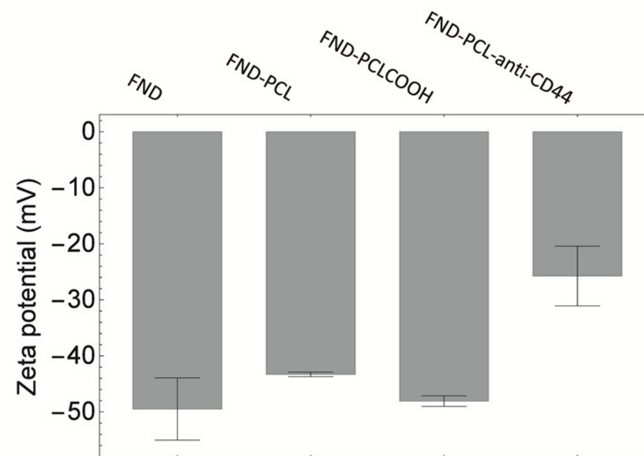

Figure S1. Zeta potentials of the samples prepared in the study.

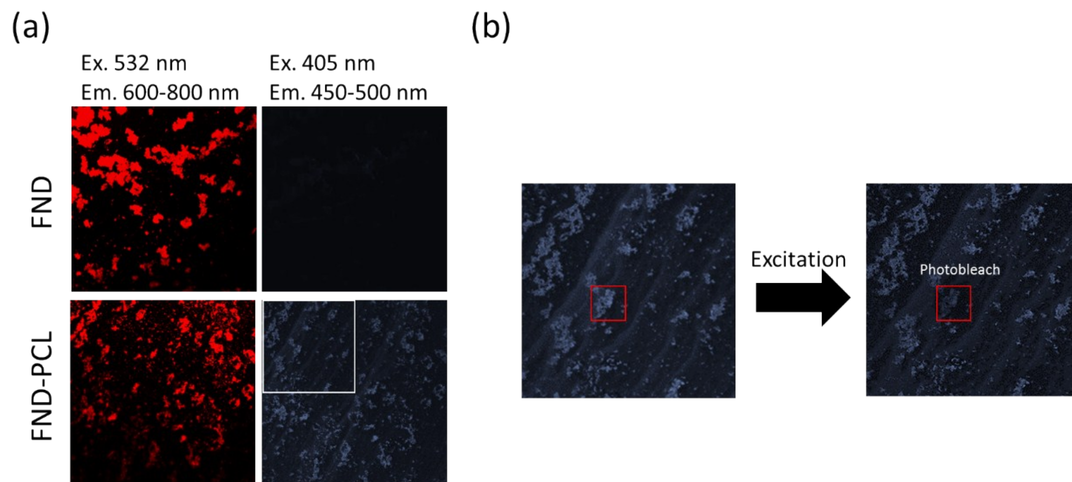

Figure S2. Fluorescence images of FND and FND-PCL deposited on glass slides. (a) Red and blue spots represent NVC fluorescence and auto-fluorescence from PCL layer, respectively. Note that the excitation condition by 405 nm is significantly strong compare to those for the observation of conventional fluorophores such as Hoechst 33342. (b) Close-up image of white square area in (a). The auto-fluorescence shows photobleaching after focused excitation indicated by red square area.

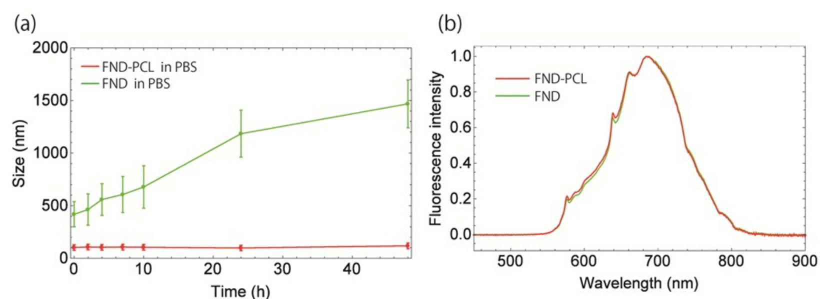

Figure S3. (a) Time-dependent size changes of the samples. Error bar indicates the standard deviation of the size distribution. (b) Fluorescence spectra of the samples.

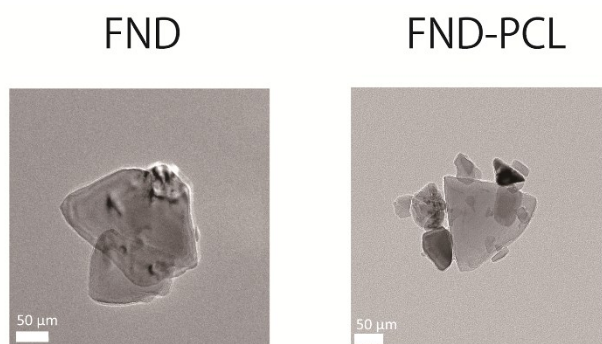

Figure S4. Typical TEM images of FND and FND-PCL. Significant difference on the surface area is not observed between them. The result implies that PCL layer is too thin and it is not observable by TEM. The images were measured using HR-TEM JEOL JEM-2010 and Tecnai G2 F20 S-TWIN (FEI) under 160 kV and 120 kV operational accelerating voltage for FND and FND-PCL, respectively.

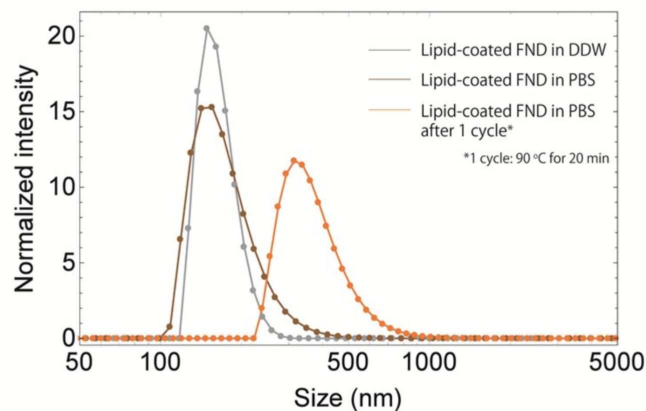

Figure S5. Thermal stability test of lipid-coated FND without crosslinking in PBS. The data show the size distributions after the one cycle of the treatment at 90 °C for 20 min. The lipid-coated FND does not show aggregation in PBS, however, they forms aggregates after the one cycle. Note that the PEG-containing lipid were used for coating to obtain stable dispersion of lipid-coated FND.

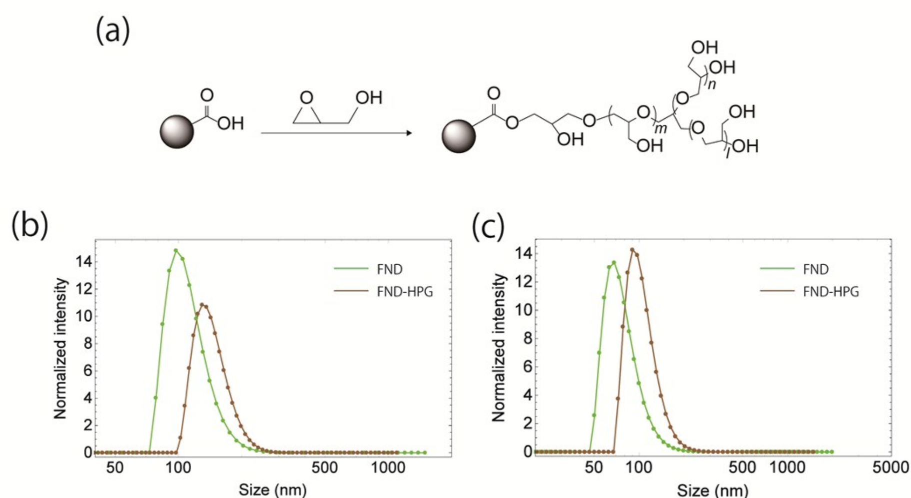

Figure S6. (a) Schematic showing for the synthesis of FND-HPG. We prepared FND-HPG according to the method reported by Zhao et.al.[2] (b) and (c) represent size changes before and after surface HPG coating using different size of FNDs. The sizes of FNDs are enlarged from 103 or 74 nm to 143 or 100 nm, respectively.

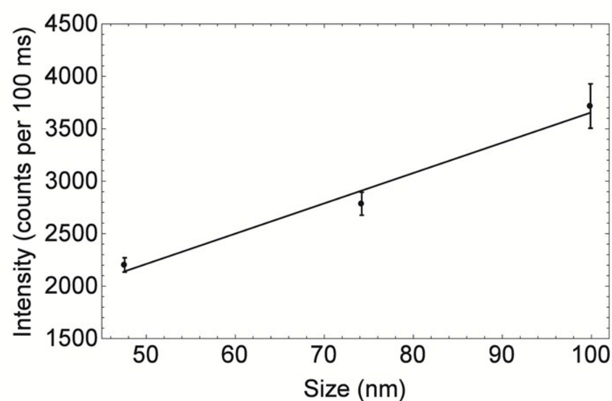

Figure S7. Size-dependent fluorescence intensity of FND. FND samples with different sizes were dispersed in DDW at a concentration of 100 μg/ml and excited by laser at 532 nm. Error bar shows the standard deviation of maximum fluorescence intensity distribution.

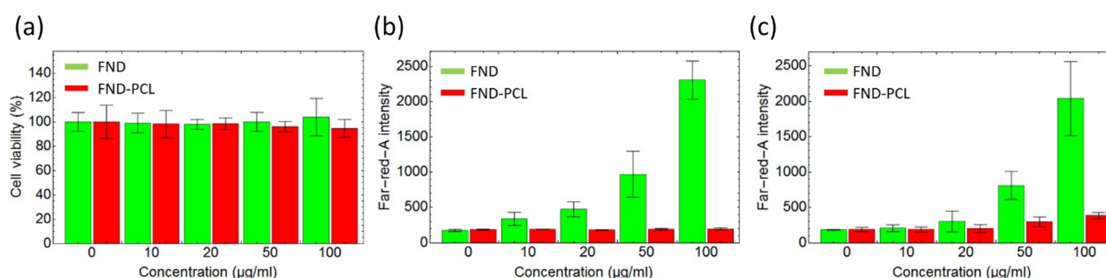

Figure S8. (a) Cytotoxicity testing of the samples on HeLa cells assayed by CCK-8 at various particle concentration of 0 – 100 μg/ml. (b,c) Flow cytometric analysis of the non-specific adsorption of the samples at different concentration on (b) live and (c) fixed HeLa cells after the incubation with FND samples. The vertical axis represent the fluorescence intensity detected in the far-red region. Values are means ± standard deviation. The reduced nonspecific adsorption, so called "stealth effect", would be achieved as a result of the covering of hydrophobic surface of FNDs by the lipids.[2]

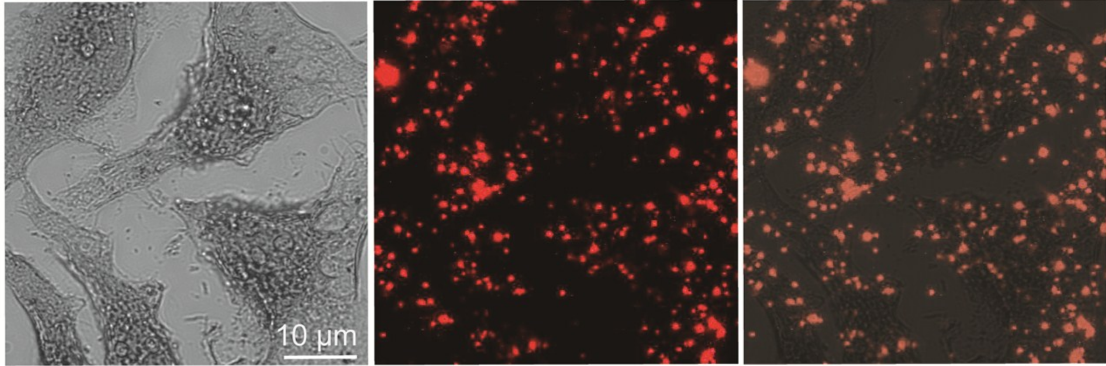

Figure S9. Bright field (left), stacked fluorescence (center), and merged image (right) of fixed HeLa cells whose CD44 membrane proteins are labelled with FND-PCL-anti-CD44.

3. Table:

Table S1. Comparison of nanodiamond coating methods

Coating	Reaction time[a]	Bonding[b]	Thickness	Specificity (Cell)	Uniformity	Ref.
PCL[c]	90 min	C	2-3 nm	++ (HeLa)	Uniform	This work
Lipid	< 1 h[d]	NC	55 nm	Unknown	Uniform	[4]
Silica	2-3 d	C	8 nm	Unknown	Uniform	[5]
Silica with PEG or copolymer	1-2 d	C	20 nm	++ (U-87 MG)	Uniform	[6],[7]
Hyperbranched polyglycerol	20 h	C	9–20 nm[e]	++ (HeLa and U87MG)	Uniform	[3],[8]
BSA	3 h	NC	~ 5 nm[f]	+ (HepG2)	Non-uniform[g]	[9]
HSA with PEG	1 d[h]	NC	11 nm	Unknown	Non-uniform[g]	[10]

[a] Coating processes only. [b] C: covalent, NC: non-covalent. [c] Photo-crosslinked lipid-coating. [d] Surface pre-treatment of FNDs is required. [e] The values are also reported in the Supporting Information. [f] Estimated from the size distributions shown in the figure. [g] Large numbers of different functional groups (COOH, NH_2, OH, SH, and etc.) from proteins are exposed. [h] Pegylated HSA is required to be prepared in advance.

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