Materials and methods

Chemicals and reagents

KOD Plus polymerase and DNase I were bought from Toyobo (JPN) and Takara (JPN), respectively. SYBR Green I and II dyes were purchased from Invirtogen (USA). 2-Oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (POPC), poly(ethylene glycol)-grafted-distealoylphosphatidylethanolamine (DSPE-PEG_{5000}), and cholesterol were purchased from Sigma-Aldrich Japan (JPN), Nippon Oil and Fat (JPN), and Wako Pure Chemical (JPN), respectively. DNA primers were purchased from Sigma-Aldrich Japan (JPN). A linear PCR template (1229 bp) coding the green fluorescent protein (GFP) was amplified from a plasmid: pQBI T7-GFP (Wako Pure Chemical, JPN) with the same primers for "PCR in GV". TE-saturated phenol-chloroform-*iso*-amylalcohol (25:24:1, *v*/*v*/*v*) and pre-cast 12.5% poly(acrylamide) gel, were purchased from Nakarai (JPN) and ATTO (JPN), respectively.

Freeze-dried empty liposome method

1) Preparation of lyophilized lipids: Stocked lipids in $CHCl_3$ were mixed in a glass tube with the following composition (POPC:DSPE-PEG_{5000}:cholesterol = 13:1:6 in mole ratio, total amount of lipid was 0.2 μmole/sample. The organic solvent was evaporated by blowing nitrogen gas to the lipid mixture to form lipid film on the inner surface of glass tube. The remaining organic solvent in the lipid film was removed under reduced pressure (20 mmHg) for 2 hr. Addition of Gibco ultra-pure water (40 μL/sample, DNase and RNase free, distilled and filtered, Invitrogen, USA) to the lipid film, followed by vortex mixing (5 sec) and sonication (5 sec) formed a vesicular dispersion (lipid concentration = 5 mM). The dispersion was frozen by liquid nitrogen, and lyophilized to obtain a freeze-dried empty liposome.

2) Preparation of GV containing PCR components: A 20 μL of real-time PCR solution [0.02 U/μL KOD Plus polymerase with anti-KOD antibody (i.e. "hot-start" enzyme for the suppression of non-specific amplification)], 0.2 nM DNA template, 0.3 μM primers, 0.2 mM dNTPs mixture, 1 mM $MgSO_4$, 1×PCR buffer (affixed to KOD Plus polymerase), and 0.5×SYBR Green I dye [λ_{ex}: 497 nm, λ_{em}: 520 nm]) was added to the freeze-dried empty liposome (0.2 μmole) on ice. Rehydration of the freeze-dried empty liposome formed GVs containing the PCR solution. We found that efficient and specific amplification of DNA was occasionally disturbed in the presence of 1×SYBR Green I which was the standard manufacturer's concentration. Since it would be an inhibition effect on the polymerase activity by the SYBR Green I dye, the concentration of the dye reduced to one half (0.5×SYBR Green I). Then the amplification efficiency became completely reproducible and the non-specific amplification was remarkably reduced.

Following gentle pipetting manipulation formed GVs encapsulating the PCR reagents, the GV dispersion was left for 30 min at room temperature in order to stabilize morphology of the GV. DNase I of 0.02 U/μL in 1×PCR buffer containing 0.5×SYBR Green I and 1 mM $CaCl_2$ instead of $MgSO_4$ was added to the GV dispersion in order to digest the DNA template and primers in exterior the GV (at room temperature, for 30 min).

Thermal cycling condition and after treatment

The GV dispersion was treated with a thermal cycler (DNA Engine Opticon 2, MJ Research, USA) under following thermal conditions: 94 °C for 2 min, [94 °C for 15 sec and 68 °C for 1.5 min] × 20 cycles (the lid contacted to the top of PCR tube was heated to 105 °C). For a flow cytometric analysis, 20 μL of the GV dispersion was prepared and separated into 10 sample tubes (20 μL each). The samples were thermally cycled in 0, 2, 4, 6, 8, 10, 12, 14, 17, and 20 times, respectively. In the end of the thermal protocol, the dispersion was slowly cooled to room temperature. Finally, DNase I was added again to the resulting GV suspension (at room temperature, for 30 min) in order to digest DNA fragments which might be leaked from the vesicle. After DNase I treatment, the GV dispersion was heated at 80 °C for 10 min to inactivate DNase I.

Phase contrast and fluorescent microscopy

Microscopic samples were prepared as follows. The thermally cycled GV dispersion was diluted to 0.2 mM as lipid concentration with an isotonic buffer (1× PCR buffer, 0.5×SYBR Green I, 1 mM $MgSO_4$, and 1% glycerol). The diluted dispersion was set between two plates of cover glass with a spacer (25×25 mm Frame Seal Chamber, MJ Research Inc., USA) which adhered to the cover glass plates in both sides. Phase contrast and fluorescent microscopic images of the GVs were obtained with the optical microscope (IX 70, Olympus, JPN) equipped with 20× objective lens and a optical filter set ($\lambda_{ex.}$: 460-490 nm, $\lambda_{em.}$: 510-550 nm).

Retrieval of PCR product in GV and poly(acrylamide) gel electrophoresis (PAGE)

A PCR product in the GV was retrieved by means of the following procedure. A mixture of 150 μL TE-saturated phenol-chloroform-*iso*-amylalcohol (25:24:1, *v*/*v*/*v*) was added to the thermally cycled GV dispersion after DNase I treatment (150 μL), and the lipids and enzymes were removed from the buffered solution containing the PCR product. After the aqueous layer was rinsed with 150 μL $CHCl_3$, remaining chloroform of the aqueous layer was removed under reduced pressure by a centrifugal evaporator. The PCR product in the resulting aqueous solution was precipitated with 70% EtOH treatment. One of the reasons why the non-specific amplification was repressed could be the usage of hot-start version DNA polymerase whose activity before the thermal cycling treatment was reduced by the anti-polymerase antibody. The purified PCR product from GV was analyzed with the

pre-cast 12.5% poly(acrylamide) gel. After electrophoresis, the gel was stained with SYBR Green II. A gel image was obtained with fluoro-image analyzer (FLA-5100, Fujifilm, JPA).

Expression of GFP using amplified 1229-bp DNA retrieved from the GV

Since the amplified DNA would code the green fluorescent protein (GFP) in our case, the GFP was expressed using the retrieved 1229-bp DNA in a cell-free translation system (PURESYSTEM classic II, Post Genome Institute, JPN) with the manufacturer's standard protocol. A time course of GFP expression and fluorescent spectra of GFP was recorded with fluorescent spectrometer. The fluorophore formation of GFP is sensitive to the mutation (change of DNA and amino acid sequences). Since fluorescence properties in Figure S1 proved expression of GFP, the mutation of GFP gene does not occur.

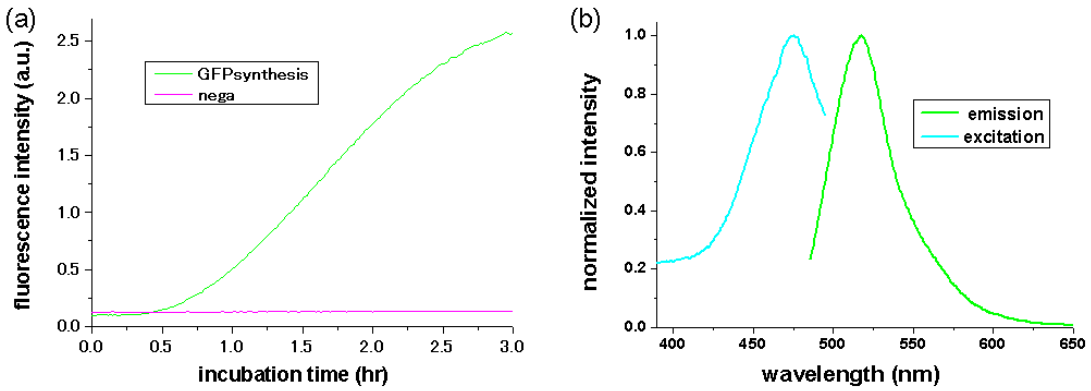

Figure S1 (a) Incubation time dependence of fluorescence intensity caused by the GFP synthesis with and without the DNA amplified in GV. (b) Fluorescence spectra of the synthesized GFP.

Flow cytometric measurement

The thermal cycled GV dispersion was diluted 50 times with the isotonic buffer (1× PCR buffer, 0.5×SYBR Green I, 1 mM $MgSO_4$, and 1% glycerol), and was passed through 40 μm-meshed filter (Falcon, USA). A sample flow was irradiated by an Ar laser (wavelength: 488 nm) equipped in a fluorescence-activated flow cytometer (Epics Altra, Beckman Coulter, USA), and the intensities of the forward scatter and fluorescence around 525 nm were collected (50,000 particles/sample). The data collection rate was controlled between 200 and 300 particles/sec.