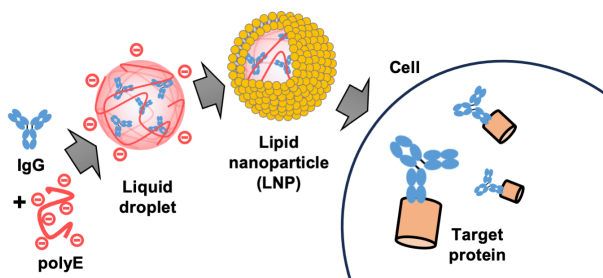


Liquid droplet-mediated formulation of lipid nanoparticles encapsulating immunoglobulin G for cytosolic delivery

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ABSTRACT: Antibodies are promising biopharmaceuticals that offer new therapeutic options for diseases. Since antibodies are membrane impermeable, approaches that allow immunoglobulin Gs (IgGs) to access intracellular therapeutic targets would open new horizons in antibody therapies. Lipid nanoparticles (LNPs) are among the classes of vectors that deliver biopharmaceuticals into cells. Using liquid droplets formed by IgG and polyglutamate, we report here a unique approach to formulate LNPs containing IgG via liquid droplets formed in the presence of polyglutamic acid (polyE). The addition of polyE promoted the formation of smaller LNPs formation with cationic lipids than in its absence, and the formed LNPs were much more efficient in cytosolic IgG delivery and targeting of cellular proteins. This approach also allows encapsulation of intact IgG without the need for chemical or sequence modification. The intracellularly delivered IgG retained its target binding ability as demonstrated by labeling of nuclear pore complex and HRas-GFP and inhibition of anti-apoptotic cell death by phosphorylated Akt protein in live cells.

KEYWORDS: liquid droplet, lipid nanoparticle, formulation, cytosolic delivery, immunoglobulin G, intracellular targeting

Introduction

Antibodies, including immunoglobulin G (IgG), are one of the most powerful modalities in current medicine due to their high target recognition capabilities.¹ The hydrophilic nature of antibodies prevents them from penetrating cell membranes. As a result, the majority of current antibody therapeutic targets are limited to those in body fluids and cell surfaces. If antibodies can target intracellular proteins, such as those involved in cancer proliferation, the scope of antibody therapy should be greatly expanded.^{2, 3} Aiming at the delivery of antibodies into cells, various systems using membrane permeant peptides, lipids and polymers have been developed to facilitate their cellular uptake and cytosolic distribution.⁴⁻¹³ However, the applicability of these approaches has mostly been demonstrated at the level of cultured cells. Due to the differences in the in vivo behavior of IgG and other materials that aid in the cellular uptake of IgG in terms of absorption, distribution, metabolism, and excretion, approaches that have been demonstrated at the level of cultured cells are often not readily translatable to IgG delivery in vivo.

There are many lipid nanoparticle (LNP) and polymer-based vectors that have already been established for in vivo delivery of nucleic acids.¹⁴ If the antibodies can be readily formulated into these vectors, the lessons learned so far can be readily applied to IgG delivery. Vectors developed for nucleic acid delivery are generally designed to be formulated via electrostatic interaction with nucleic acids, and negatively charged proteins can be encapsulated in LNPs for translocation into cells.¹⁵ However, the isoelectric point of most IgGs has been reported to be in the range of 6.4-9.0,¹⁶ and therefore IgGs are generally less amenable to formulation into nucleic acid delivery vectors. Although chemical modification of IgGs is one possible approach to add negative charges to IgGs, this requires optimization of the physicochemical properties of the resulting IgGs to avoid

potential aggregate/precipitate formation and impairment of antigen recognition capabilities. Therefore, approaches to effectively formulate intact IgGs into vectors have been awaited.

Here, we focused on an approach to concentrate IgG by liquid-liquid phase separation into liquid droplets by mixing IgG with negatively charged polyglutamic acid (polyE). Liquid-liquid phase separation (LLPS) and liquid droplet formation have attracted considerable research interest in the fields of cell biology and drug delivery.^{17, 18} We and others have also demonstrated the intracellular delivery of antibodies and mRNAs with marked efficacy using lipid droplets,^{12, 19} demonstrating their promise for intracellular delivery of biopharmaceuticals. Assuming that the isoelectric point of the most IgGs is in the range of 6.4-9.0,¹⁶ they are generally positively charged at acidic pH. It has been reported by Shiraki and coworkers that liquid droplets of IgGs are formed in acidic buffer at pH 5 by electrostatic interaction with polyE²⁰ – considering that the pK_a of the side chain carboxy group of glutamic acid is 4.3, polyE should be negatively charged at pH 5. Therefore, we expected the encapsulation of IgGs into LNPs in the presence of polyE by the formation of negatively charged liquid droplets (Figure 1). In this study, we aimed to show the validity of using polyE-mediated liquid droplet for encapsulation of IgGs in LNPs – Complexation of IgG with polyE through liquid droplets facilitated the formation of more compact LNPs than in the absence of polyE, resulting in efficient cytosolic IgG delivery and targeting of cellular proteins.

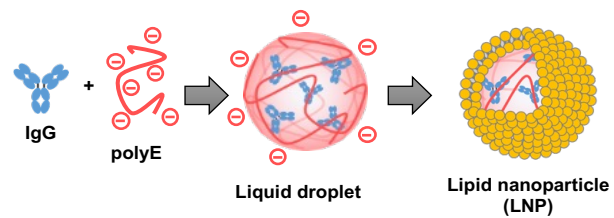


Figure 1. Concept of liquid droplet mediated encapsulation of intact immunoglobulin G (IgG) in lipid nanoparticles (LNPs) without the need for chemical and sequence modification.

Experimental Section

Materials

SS-cleavable and pH-responsive lipid, COATSOME SS-OP (SS-OP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and 1,2-dimyristoyl-rac-glycerol-3-methylpolyoxyethylene-polyethylene glycol chain (molecular weight 5000; DMG-PEG5k) were purchased from NOF Corporation. Poly-L-glutamic acid sodium salt (molecular weight 3,000-15,000) (polyE) and β -sitosterol were purchased from Sigma-Aldrich. Alexa Fluor 488 5-SDP (sulfodichlorophenol) ester and Alexa Fluor 594 NHS ester (*N*-hydroxysuccinimide ester) were from Thermo Fisher Scientific. Human immunoglobulin B, polyclonal (IgG) was from Fujifilm Wako Pure Chemical Cooperation, and anti-nuclear pore complex (NPC) IgG (anti-NPC-IgG) (Mab414, Cat#: 902902) was from BioLegend. Anti-phospho-AKT1(Ser473)-IgG (anti-pAkt1-IgG, Cat#: 700392) was purchased from Invitrogen. PD-10 column was purchased from Cytiva. Other reagents were purchased from Fujifilm Wako Pure Chemical Cooperation unless otherwise noted. Human IgG, polyclonal was used throughout this study, unless otherwise indicated, and described as IgG.

Plasmid encoding anti-GFP-IgG-HiBiT

The synthesized DNA fragment of coding HiBiT tag (5'-gagcttctcccgactccgggtaaagggtggtggatcaggaggaggtagtgttagtggatggagactattcaagaagatctcgggaggtggctcatatccgtatgatgttccggattatgcaaccggtgatctagacacgtgattaattaag-3') was inserted into the 3' end of the coding Fc domain of the anti-GFP_P1316-IgG2a plasmid (Addgene, 114492) by HiFi assembly according to the manufacturer's instructions.

Preparation of anti-GFP-IgG and anti-GFP-HiBiT

Anti-GFP-IgG and anti-GFP-HiBiT were prepared using the Expi293TM expression system (Thermo Fisher Scientific). Plasmid encoding anti-GFP-IgG (Addgene, 114492) (25 µg) or anti-GFP-HiBiT was mixed with ExpiFectamine 293 (Thermo Fisher Scientific) and transfected into Expi293F cells (3,000,000 cells/mL, 25 mL) according to the manufacturer's protocol. Twenty hours after transfection, 150 µL ExpiFectamine293 Transfection Enhancer 1 and 1.5 mL Enhancer 2 were added and incubated for five days. The culture medium was transferred to a 50 mL tube, centrifuged at $3,200 \times g$ for 20 min at 4°C, and the supernatant was collected. The antibody-containing supernatant was sterilized by filtration through a 0.22 µm filter. The solution was then applied to HiTrap Protein A High Performance (Cytiva). IgG was released from the column by a pH gradient in purification buffer (0.1 M glycine buffer, pH = 2.7), and the buffer of the fraction containing anti-GFP-IgG was exchanged to PBS(–) by dialysis. The resulting anti-GFP-IgG was stored at 4°C.

Preparation of AF488 labeled IgG [IgG(AF488)] and AF594 labeled IgG [IgG(AF594)]

Human IgG, polyclonal (10 mg/mL, 100 µL) was diluted with PBS(–) (345 µL). After sequential addition of 1 M NaHCO₃ (50 µL) and Alexa Fluor 488-5 SDP ester in DMSO (10 mg/mL, 10 µL), the mixture was gently stirred at 25°C for 1 h and subjected to gel-filtration [Sephadex G-25 (Medium)] on a PD-10 column. IgG(AF594) was prepared similarly using Alexa594-NHS ester instead of Alexa Fluor 488-5 SDP ester.

LNPs encapsulating IgGs

Poly-L-glutamic acid (polyE) (0.75 mg/mL) in 10 mM citrate (pH 5.0) was incubated at 40°C for 30 min. IgG (10 mg/mL = ~66 µM) in PBS(–) was added at a mass ratio of IgG:polyE = 5:1

and pipetted 10 times to obtain liquid droplets. A solution of a mixture of SS-OP, DOPE, β -sitosterol, DMG-PEG5k in t-BuOH (molar ratio = 45/10/45/1.5, total lipid concentration = 4 mM) incubated for 30 min at 40°C was added to the solution containing liquid droplets at a lipid/liquid droplet volume ratio of 1:4 and gently mixed by pipetting 15 times. The mixture was then dialyzed more than 1000-fold against PBS(–) using Spectra/Por (MWCO 12-14k, Spectrum Laboratories Inc.) to obtain LNPs encapsulating IgGs. LNPs encapsulating IgG(AF488), IgG(AF594), anti-GFP-IgG, anti-GFP-IgG-HiBiT, anti-NPC-IgG, and anti-phospho-AKT1(Ser473)-IgG (anti-pAkt1-IgG) with the help of polyE were similarly prepared as stated above.

Zetasizer Nano ZS (Malvern) was used to analyze the size, polydispersity index (PDI), and zeta potential of LNPs. Results are presented as the mean $\pm$ standard deviation (SD) of more than three independent experiments (Table S1).

Encapsulation efficiency of IgG in LNPs

LNPs formulated with IgG in the presence of polyE in PBS(–) (100 μ L, IgG concentration was adjusted to 2 μ M) was centrifuged at 20,000 $\times$ g for 1 h at 4°C to sediment LNP fraction. The supernatant (50 μ L) was removed for UV analysis at 280 nm.

Encapsulation efficacy was analyzed according to the following equation:

$$\text{Encapsulation efficiency (\%)} = (A_{280(\text{IgG-super})} - A_{280(\text{LNP-super})}) / A_{280(\text{IgG-super})} \times 100$$

where $A_{280(\text{IgG-super})}$ and $A_{280(\text{LNP-super})}$ represent absorptions at 280 nm of supernatants of 2 μ M IgG and [IgG_polyE]LNP centrifuged at 20,000 $\times$ g for 1 h at 4 °C.

Cell culture

HeLa cells, a human epithelial carcinoma cell line, were obtained from the European Collection of Authenticated Cell Cultures (ECACC) (93021013) and cultured in α -minimum essential medium containing 10% (v/v) heat-inactivated bovine serum [α -MEM(+)] under a 5% CO₂ atmosphere at 37°C and passaged every 2-4 days.

Flow cytometry

HeLa cells were seeded at 80,000 cells/well in 24-well plates (Iwaki) and incubated overnight. The cells were washed twice with PBS(–) and then incubated with 20 μ M chlorpromazine (CPZ), 80 μ M EIPA, 500 nM wortmannin, and 20 μ M nystatin in α -MEM(–) for 30 min at 37°C in the presence of 5% CO₂, respectively. After incubation, the medium was replaced with α -MEM (+) containing [IgG(AF488)_polyE]LNP (0.5 μ M IgG concentration) and inhibitors and incubated at 37°C for 1 h under 5% CO₂. The cells were then washed twice with PBS(–), treated with 0.01% trypsin for 10 min and centrifuged (800 $\times$ g, 5 min). The cells were then washed twice with PBS(–) and resuspended in 500 μ L of PBS(–). Fluorescence from 10,000 cells per sample was quantified using an Attune NxT flow cytometer (Invitrogen).

Confocal microscopy

HeLa cells (1.2×10^5 cells) were seeded on 35 mm glass-bottom dishes (inner diameter ~34 mm, observation well size 12 mm) (Iwaki) and incubated at 37 °C with 5% CO₂ overnight. After two washes with PBS(–), cells on the observation well ($\sim 1.5 \times 10^4$ cells) were treated with [IgG(AF488)_polyE]LNP in α -MEM(+) (200 μ L) at an IgG concentration of 0 - 1 μ M, and incubated at 37°C under 5% CO₂ for 18 h. The cells were then washed twice with PBS(–) and incubated in α -MEM(+) for 6 h at 37°C under 5% CO₂. Cells were washed twice with PBS(–),

nuclear stained with 5 $\mu\text{g/mL}$ Hoechst33342 (Thermo Fisher Scientific), and the medium was changed to $\alpha\text{-MEM}(+)$. Live cell imaging of the subcellular localization of IgG(AF488) was performed using an FV1000 or FV3000 confocal laser microscope (Olympus).

For immunostaining, cells were fixed with 4% paraformaldehyde at 25°C for 10 min. Cells were then washed with PBS(–) and permeabilized with 0.1% Triton in PBS(–). Cells were incubated with 1% bovine serum albumin (BSA) in PBS containing 0.5 w/v% Tween20 (PBS-T) for 30 min at 25 °C for blocking, and then treated with secondary antibodies (Table S2). After washing with PBS-T, PBS(–) was added and the localization of antibodies was analyzed by CLSM.

For time-course observation, HeLa cells were incubated in $\alpha\text{-MEM}(+)$ containing [IgG_polyE]LNP (IgG concentration, 1 μM) at 37°C under 5% CO₂ for 1, 3, 6, 9, and 12 h. Cells were washed with PBS(–) and medium was changed to $\alpha\text{-MEM}(+)$ prior to CLSM observation.

For the cellular uptake experiment at 4°C, HeLa cells were preincubated in $\alpha\text{-MEM}(+)$ for 30 min at 4°C. The medium was replaced with $\alpha\text{-MEM}(+)$ containing [IgG_polyE]LNPs (IgG concentration, 1 μM) and incubated for 6 h at 4 °C. Cells were washed twice with PBS(–) and the medium was replaced to $\alpha\text{-MEM}(+)$ prior to CLSM observation.

Galectin-8 assay

HeLa cells (1.2×10^5 cells) were seeded on 35-mm glass-bottom dishes and transfected with a plasmid encoding Gal8-GFP constructed by GenScript using Lipofectamine LTX (Thermo Fisher Scientific) according to the manufacture's protocol. Twenty-four hours after transfection, the cells were washed twice with PBS(–) and incubated with [IgG_polyE]LNP (IgG concentration, 1 μM) in $\alpha\text{-MEM}(+)$ for 18 h at 37°C under 5% CO₂. After incubation, the cells were washed twice with

PBS(–) and the medium was replaced with α -MEM(+) prior to observation of Gal8-GFP signals by CLSM.

Delivery of anti-GFP-IgG to HRas-GFP expressing HeLa cells

HeLa cells were transfected with plasmids encoding HRas(G12V)-GFP (Addgene, 18666) using Lipofectamine LTX (Thermo Fisher Scientific) according to the manufacturer's protocol. After washing twice with PBS(–) 24 h after transfection, the cells were incubated with [anti-GFP-IgG_polyE]LNP (IgG concentration, 1 μ M) in α -MEM(+) at 37°C for 18 h under 5% CO₂. Cells were washed twice with PBS(–) and the medium was changed to α -MEM(+). Signals from HRas(G12V)-GFP and anti-GFP-IgG were observed by CLSM.

To observe the cellular localization of anti-GFP-IgG without fluorescence labeling, cells were fixed after incubation, immunostained with a secondary antibody, anti-mouse-IgG-AF568, and their localization observed by CLSM.

Evaluation of the delivery efficiency of [anti-GFP-IgG-HiBiT_polyE]LNP to LgBiT-expressing HeLa cells

LgBiT-expressing HeLa cells were seeded at 50,000 cells/well in 24-well plates (Iwaki) and incubated overnight. The cells were washed twice with PBS(–) and incubated with [anti-GFP-IgG-HiBiT_polyE]LNP (IgG concentration, 0.5 μ M) in α -MEM(+) for 18 h at 37°C under 5% CO₂. The cells were then washed twice with PBS(–), treated with 0.01% trypsin for 10 min and centrifuged (800 $\times$ g, 5 min). Cells were then washed twice with PBS(–) and resuspended in 75 μ L of PBS(–) followed by the addition of 25 μ L Nano-Glo® Live Cell Reagent (Promega) diluted

20-fold. Cells were immediately transferred to 96-well white plates (PerkinElmer) and the chemiluminescence was quantified using a Nivo multimode plate reader (PerkinElmer).

When the unencapsulated antibody was removed by Protein A agarose beads, 10 μ L Protein A agarose beads (Ab-Capcher, ProteNova) were washed three times with PBS, added to [anti-GFP-IgG-HiBiT_polyE]LNP and rotated for 30 min at room temperature. The supernatant containing LNP was diluted to α -MEM(+) and added to LgBiT-expressing HeLa cells, followed by 18 h incubation at 37°C under 5% CO₂. The chemiluminescence measurement protocol was the same as described above.

Delivery of anti-NPC-IgG

HeLa cells (1.2×10^5 cells/well) were seeded on 35-mm glass-bottom dishes (Iwaki) and incubated overnight at 37 °C with 5% CO₂. Cells were then treated with [anti-NPC-IgG_polyE]LNP (IgG concentration, 1 μ M) in α -MEM(+) at 37 °C with 5% CO₂ for 18 h. Cells were washed twice with PBS(–) and stained with 5 μ g/mL Hoechst33342. Cells were then fixed and immunostained with anti-mouse IgG-AF568, a secondary antibody, prior to observation with CLSM. The percentage of cells in which fluorescent signals from secondary antibodies recognizing anti-NPC IgG were observed was counted from ~400 cells.

Induction of apoptosis by anti-pAkt1-IgG

HeLa cells (4×10^3 cells/well) were seeded on 96-well white plates (Thermo Fisher Scientific) and incubated overnight at 37 °C with 5% CO₂. Cells were then incubated with [anti-pAkt1-IgG_polyE]LNP or [IgG_polyE]LNP (IgG concentration, 1 μ M) in α -MEM(+) (100 μ L) at 37 °C with 5% CO₂ for 24 h. Caspase-Glo 3/7 Assay Reagent (Promega) (100 μ L) was added and cells

were gently shaken with shading for 1 h. Luminescence was analyzed using a VICTOR Nivo Multimode Microplate Reader (PerkinElmer).

Statistical Analysis

Statistical analyses were performed with JMP Pro (ver. JMP 15.1.0, SAS Institute). All experiments were repeated in triplicate. Data are presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using unpaired two-tailed Student's t-test for two groups and one-way ANOVA with Tukey's test for multiple comparisons. Differences in the compared groups were considered significantly different when $P < 0.05$. *, **, and *** indicate P values less than 0.05, 0.01, and 0.001, respectively.

Results and discussion

Liquid droplet-mediated formulation of lipid nanoparticles

Considering the high efficacy in nucleic acid delivery,²¹ a commercially available pH-responsive lipid, COATSOME SS-OP (SS-OP) (Figure S1), was used as the lipid for LNP formation. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and β -sitosterol²² were used as helper lipids for easy formulation of LNP structures and transfection (subcellular transport) of LNP contents. The lipid composition on the LNP preparation was set as SS-OP/DOPE/ β -sitosterol = 45/10/45 (molar ratio), referring to our previous reports and others.^{15, 21-23} For LNP surface decoration with polyethylene glycol (PEG) to prevent aggregation of LNP, 1,2-dimyristoyl-*rac*-glycerol-3-methylpolyoxyethylene-polyethylene glycol chain (molecular weight 5000; DMG-PEG5k) was also added at 1.5 mol% of the total lipid.

Alexa Fluor 488 (AF488)-labeled IgG (human, polyclonal) [IgG(AF488)] in phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(-)] was mixed with polyE in 10 mM citrate buffer (pH 5.0) (IgG:polyE = 5:1 w/w), and complexes or liquid droplets with diameters of a few μm were formed as reported ([IgG(AF488)_polyE], Figure 2A),^{20, 24} using AF488 labeling to visualize lipid droplet formation and cellular uptake of IgG. [IgG(AF488)_polyE] was then mixed with the above lipid mixture in *t*-butanol (*t*-BuOH) at a molar ratio of IgG:lipid = 1:200, followed by dialysis of the mixture against PBS(-) to remove excess *t*-BuOH. An IgG-containing LNP preparation ([IgG(AF488)_polyE]LNPs) was thus obtained. The encapsulation of IgG(AF488) in LNPs was suggested by the colocalization of the IgG(AF488) signal with that of LNPs labeled with rhodamine-conjugated phosphatidylethanolamine (Rho-PE) (Figure 2B) – in particular, this procedure was accompanied by the disappearance of liquid droplet structures of a few μm diameter, but the formation of dot-like structures of much smaller size with both AF488 and rhodamine

signals. Dynamic light scattering analysis of the IgG-containing LNPs formed in the presence of polyE suggested the formation of particles with diameters of ~ 300 nm (Tables S1). Further studies are needed to rationalize why the size of the liquid droplets was reduced by the formation of LNPs. One possible explanation would be that the IgG complexed with polyE in the liquid droplets was more condensed by the interaction of the cationic lipid mixture and the LNPs of smaller size were formed.

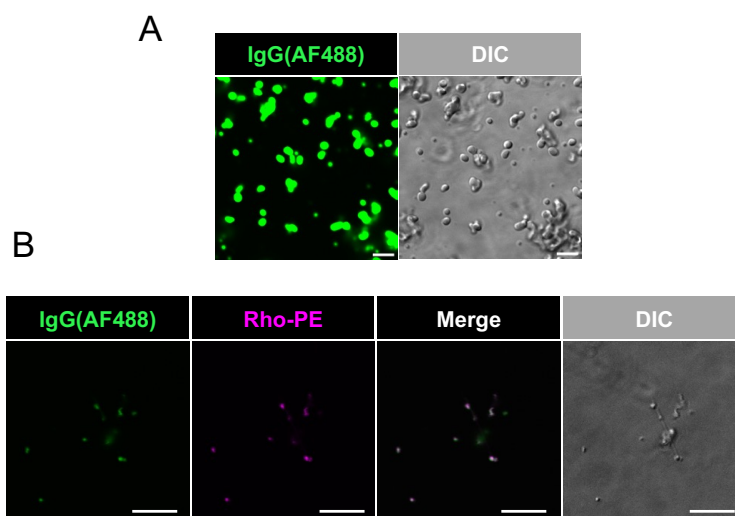


Figure 2. Observation of (A) [IgG(AF488)]_polyE droplet and (B) [IgG(AF488)]_polyE LNPs by confocal laser scanning microscopy (CLSM). In (A) Alexa Fluor 488 labeled IgG (human, polyclonal) [IgG(AF488)] were mixed with polyE in 10 mM citrate buffer (pH 5) at mass ratio of IgG:polyE = 5:1. In (B) the [IgG(AF488)]_polyE droplet was mixed with lipids (SS-OP/DOPE/ β -sitosterol/DMG-PEG5k/Rhodamine-PE (Rho-PE) = 45/10/45/1.5/0.1 (molar ratio)) and dialyzed against PBS(–). Scale bar, 5 μ m.

Cytosolic delivery of IgG

Cytosolic delivery of IgG was confirmed by confocal laser scanning microscopy (CLSM) analysis after treatment of HeLa cells with [IgG(AF488)_polyE]LNPs for 18 h. Diffuse signals of AF488 throughout the cells suggest the cytosolic distribution of IgG(AF488) (Figure 3A, LNP). In contrast, cells treated with IgG(AF488) alone or the liquid droplet formed by IgG(AF488) with polyE [IgG(AF488)_polyE] yielded only punctate signals, suggesting predominant endosomal entrapment of IgG(AF488) (Figure 3A, IgG alone and liquid droplet).

LNP formulation using liquid droplet had a significant effect on cytosolic IgG delivery. In marked contrast to cellular treatment with [IgG(AF488)_polyE]LNPs, treatment with LNPs similarly prepared in the absence of polyE ([IgG(AF488)]LNPs) was associated with little cytosolic appearance of IgG(AF488) signals (Figure 3B). Flow cytometry analysis indicated that [IgG(AF488)_polyE]LNPs delivered almost twice the amount of IgG(AF488) into cells than [IgG(AF488)]LNPs (Figure 3C).

The validity of LNP formulation using liquid droplet of IgG with polyE was further confirmed using a HiBiT/LgBiT split luciferase system (Figure 3D, Figure S2).²⁵ Recombinant anti-GFP-IgG bearing a HiBiT tag at the C-terminus of the heavy chain was thus prepared as a model IgG (anti-GFP-IgG-HiBiT). Cytosolic translocation of anti-GFP-IgG-HiBiT in LgBiT-expressing cells leads to reconstitution of the split luciferase (NanoLuc luciferase; K_d of HiBiT peptide with LgBiT protein segment = 700 pM)²⁵ and cytosolic delivery efficiency can be evaluated by luciferase activity (Figure S2). The luminescence generated by treatment with LNP formed with anti-GFP-IgG-HiBiT in the presence of polyE ([anti-GFP-IgG-HiBiT_polyE]LNP) (correlating cytosolically

delivered protein amounts) was 7-fold higher than that formed in the absence of polyE ([anti-GFP-IgG-HiBiT]LNP), indicating the impact of polyE on cytosolic IgG delivery (Figure 3D).

Time course observation of cells treated with [IgG(AF488)_polyE]LNPs revealed a gradual increase in diffuse cytosolic IgG(AF488) signals, suggesting the contribution of endocytosis to cellular uptake (Figure 3E); endosomal maturation is accompanied by a decrease in pH in the endosomal lumen, leading to LNP-mediated endosomal destabilization and cytosolic release of encapsulated IgG(AF488).

The involvement of endocytosis in the cellular uptake of IgG(AF488) was further confirmed by the absence of cellular IgG(AF488) signals after treatment with [IgG(AF488)_polyE]LNPs at 4°C (Figure 4A); endocytosis is an ATP-driven cellular machinery and does not operate at this temperature.²⁶ A possible involvement of clathrin-mediated endocytosis and macropinocytosis in the endocytic uptake of [IgG(AF488)_polyE]LNPs was also suggested by the inhibitor studies (Figure 4B). Rupture of the endosomal membrane by LNPs encapsulating IgG in the presence of polyE was further confirmed by galectin-8 assay (Figure 4C).²⁷ Galectin-8 is a β -galactoside-binding protein. Rupture of endosomal/lysosomal membranes allows galectin-8 to enter and bind to β -galactosides present on the luminal side of endosomal membranes. Treatment of the cells expressing galectin-8 fused to an enhanced green fluorescent protein (EGFP) with [IgG_polyE]LNPs resulted in prominent punctate signals of galectin-8-EGFP (arrowheads) in the cells, indicating ruptured endosomal membranes.

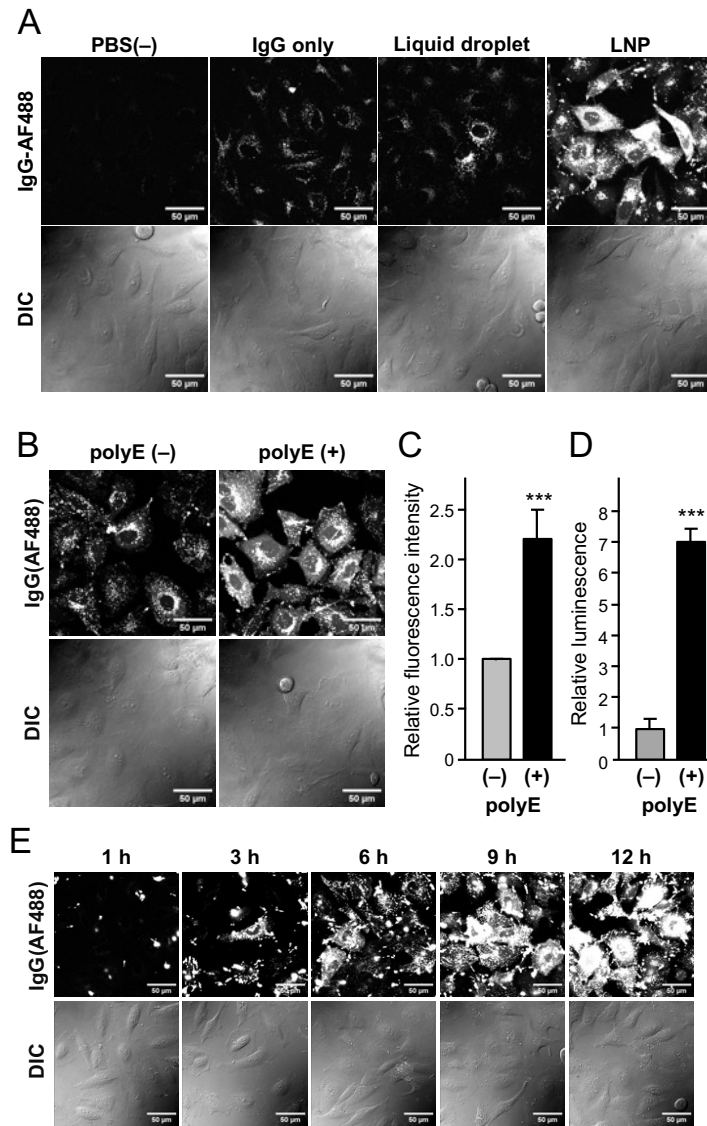


Figure 3. (A) Marked cytosolic distribution of IgG(AF488) in the cells treated with [IgG(AF488)_{polyE}]LNPs for 18 h. (B-E) Role of polyE for cytosolic distribution of IgG(AF488) analyzed by (B) CLSM observation, (C) flow cytometry, and (D) HiBiT/LgBiT split luciferase system. (E) time-dependent increase of cytosolic IgG(AF488) distribution.

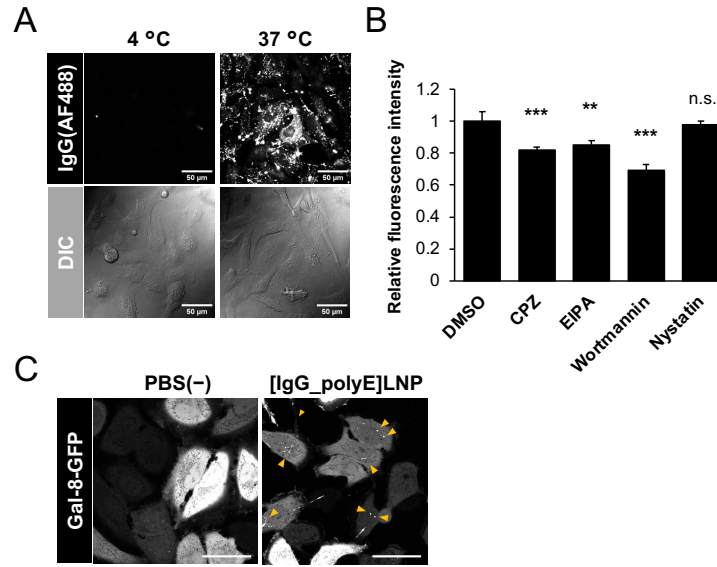


Figure 4. (A) CLSM observation of IgG(AF488) signals after treatment with [IgG(AF488)_polyE]LNP (1 μ M as IgG concentration) for 6 h at 4 and 37 °C. (B) Total cellular uptake of IgG(AF488) in the presence of endocytosis inhibitors: 20 μ M of chlorpromazine (CPZ, a clathrin-mediated endocytosis inhibitor), 80 μ M of EIPA (a macropinocytosis inhibitor), 0.5 μ M of Wortmannin (a macropinocytosis related phosphatidylinocitol-3-kinase inhibitor) and 20 μ M of Nystatin (a caveolae-mediated endocytosis inhibitor). Cells were incubated with [IgG(AF488)_polyE]LNP (0.5 μ M as IgG concentration) for 1 h prior to flow cytometer analysis. Results were presented as the mean $\pm$ SD (n = 3). n.s., not significant; **P < 0.01; ***P < 0.001 (ANOVA followed by Dunnett's post hoc test vs. dimethyl sulfoxide (DMSO)). (C) Rupture of endosomal/lysosomal membranes suggested by puncta of galectin-8-GFP signals after treatment with [IgG_polyE]LNPs (1 μ M as IgG concentration) for 18 h.

Effect of polyE on LNP formulation and cytosolic IgG delivery

Complex formation of IgG with polyE led to concentration of IgG in liquid droplet and the formulation of compact and uniform LNPs, facilitating their cellular uptake (Table S1). LNPs formulated in the absence of polyE ([IgG]LNPs) had a diameter of ~645 nm, considerably larger than the ~294 nm of LNPs formulated in the presence of polyE ([IgG_polyE]LNPs (analyzed by dynamic light scattering); the polydispersity index (PDI) of [IgG]LNPs (0.605) was also larger than that of [IgG_polyE]LNPs (0.392). Encapsulation efficiency of IgG in [IgG_polyE]LNPs, analyzed by ultracentrifugation of LNPs followed by calculation of the amount of free, unencapsulated IgG in the supernatant based on absorbance at 280 nm, was ~34%. This value was almost the same as that of LNPs formulated in the absence of polyE ([IgG]LNPs) (Table S1). Considering that the total luminal volume of smaller diameter vesicles prepared from the same amount of lipid would be smaller than that of larger vesicles,⁸ this suggested that the luminal concentration of IgG in [IgG_polyE]LNPs was higher than that in [IgG]LNPs, further supporting the formation of liquid droplets with polyE to effectively concentrate IgG in LNPs. Considering that polyE should not have a strong membrane disrupting activity, the cytosolic release of encapsulated IgGs was achieved with the help of lipids from LNPs. The zeta potentials of these LNPs were almost identical (i.e., -6.6 mV for [IgG]LNPs and -5.6 mV for [IgG_polyE]LNPs).

We also confirmed that IgG(AF488) in [IgG(AF488)_polyE]LNPs played a key role in the cytosolic distribution by using the purified LNP preparation in which unencapsulated IgGs were removed by ultracentrifugation. Similar to the [IgG(AF488)_polyE]LNP treatment without ultracentrifugation, significant cytosolic IgG(AF488) signals were obtained by cellular treatment with sedimented [IgG(AF488)_polyE]LNPs for 18 h (Figure 5A). Quantification of internalized IgG using the HiBiT/LgBiT split luciferase system further supported the validity of LNP-

encapsulated IgG in cytosolic translocation. LgBiT-expressing HeLa cells were incubated with the [anti-GFP-IgG-HiBiT_polyE]LNP preparation, from which free, unencapsulated anti-GFP-IgG-HiBiT was removed by protein A agarose beads, for 18 h, resulting in a luminescence intensity of ~60% compared to the [anti-GFP-IgG-HiBiT_polyE]LNP preparation without removal of free anti-GFP-IgG-HiBiT (Figure 5B). These results suggest that the predominant cytosolic activity of the delivered IgG should come from those encapsulated in LNPs. Although little change was observed in the diameter, PDI, and zeta potential of LNPs before and after the protein A agarose bead purification step (Table S3), suggesting aggregation and damage of LNPs, we performed the following experiments without isolating LNPs from unencapsulated IgG in the LNP preparations for simplicity.

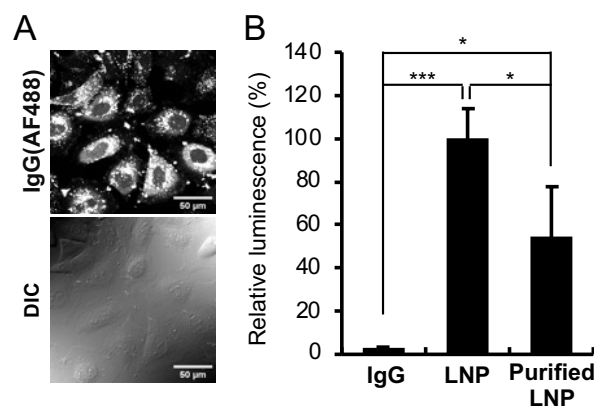


Figure 5. (A) Cytosolic appearance of IgG(AF488) by treatment with [IgG(AF488)_polyE]LNPs free of unencapsulated IgG(AF488) signals. Scale bars, 50 μ m. (B) Cytosolic translocation of IgG analyzed by HiBiT/LgBiT split luciferase assay (IgG: anti-GFP-IgG-HiBiT 1 μ M; LNP: [anti-GFP-IgG-HiBiT_polyE]LNP [total anti-GFP-IgG-HiBiT, 1 μ M]; purified LNP = aliquot of [anti-GFP-IgG-HiBiT_polyE]LNP obtained after protein A bead treatment).

The targeting ability of the intracellularly delivered IgG

We next demonstrated that fractions of IgGs thus delivered into cells retain their target recognition capabilities. This was demonstrated by the delivery of anti-nuclear pore complex (NPC) IgG (anti-NPC-IgG) encapsulated in the LNPs formulated in the presence of polyE ([anti-NPC-IgG_polyE]LNPs) (Figure 6A). Cells treated with [anti-NPC-IgG_polyE]LNPs for 18 h and immunostained with Alexa Fluor 568 (AF568)-labeled anti-mouse IgG (goat) showed prominent AF568 signals on nuclear membranes (arrowheads in Figure 6A, line-intensity profile in Figure 6B).

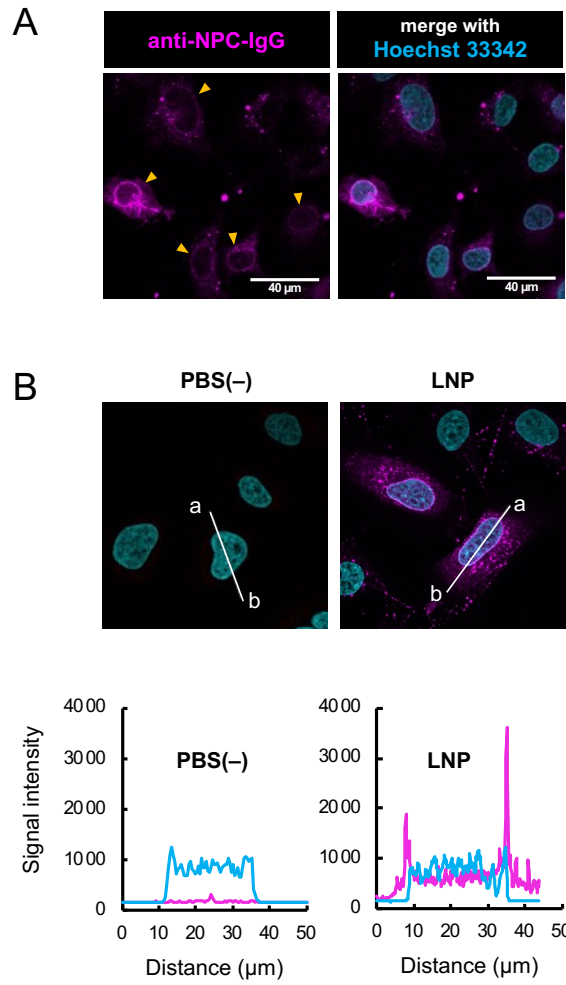


Figure 6. Recognition of intracellular targets by anti-nuclear pore complex (NPC) IgG. Scale bars, 40 μm . (B) The line intensity profile (lower panels) of nuclear signals (Hoechst33342, cyan) and IgG signals (anti-mouse IgG-secondary IgG-AF568, magenta) from a to b in CLSM images (upper line) of cells treated with PBS(-) only (left) and [anti-NPC-IgG_polyE]LNPs (right), respectively. A marked accumulation of anti-mouse secondary IgG(AF568) (secondary antibody) in the periphery of the nucleus (with Hoechst33342 signal) strongly suggested the binding of anti-NPC-IgG to NPCs.

The targeting ability of the intracellularly delivered IgG was also demonstrated by colocalization with the HRas-GFP fusion protein (Figure 7A). HRas is a member of the Ras protein family that localizes to the plasma membrane.²⁸ LNPs encapsulating anti-GFP-IgG(AF594) via a liquid droplet formed with polyE were similarly prepared; no significant differences in liquid droplet and LNP formation were observed using Alexa Fluor 488 and 594. The strong colocalization of HRas-GFP signals at the plasma membrane (indicated by white signals in the merged image in Figure 7A) with those of anti-GFP-IgG(AF594) suggested the feasibility of this approach for live cell imaging.

The potential of this approach to modulate biological signaling in cells was also exemplified by the induction of apoptosis by inhibiting Akt phosphorylation (Figure 7B). Akt is a Ser/Thr kinase and plays a central role in the PI3K/Akt signaling pathway, which regulates cell survival, proliferation, growth, apoptosis, and glycogen metabolism.²⁹ Phosphorylation of Akt suppresses apoptosis-inducing factors such as Bad, Bax, and caspase-9, thereby inhibiting apoptosis for cancer survival and promoting proliferation.³⁰ Thus, Akt is considered a therapeutic target for cancer (Figure 7B, left). Treatment of cells with LNPs containing anti-pAkt1 IgG (which recognizes phosphorylated Akt) prepared in the presence of polyE [i.e., (anti-pAkt1-IgG_polyE)LNPs] resulted in ~4-fold higher activity of caspase-3/7 (representative apoptosis mediators) than cells treated with control LNPs prepared with IgG that does not specifically target pAkt [(hIgG_polyE)LNPs] (Figure 7B, right). The activity was comparable to that reported for gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor commonly used to treat non-small

cell lung cancer,³¹ demonstrating the feasibility of this LNP-mediated intracellular IgG delivery to modulate cellular signaling.

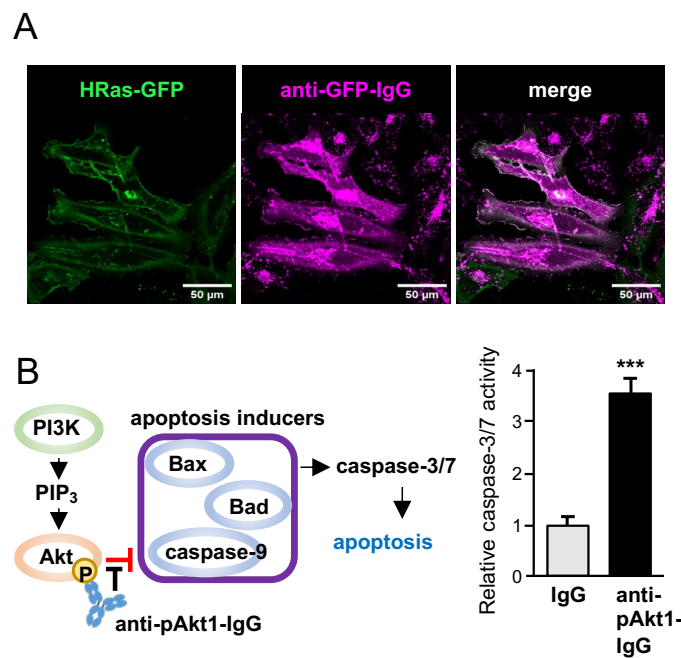


Figure 7. Recognition of intracellular targets by (A) anti-GFP IgG (AF594). (B) (Left) IgG binding to phosphorylated Akt activates apoptosis inducers. (Right) Apoptosis induction was assessed by caspase-3/7 activity assay based on luminescence (see Method). Scale bars, 50 μ m.

In this report, we have demonstrated the validity of our approach to use liquid droplets formed by IgGs and polyE for the effective encapsulation of IgGs in LNPs, where polyE played a role in forming smaller LNPs than in its absence, yielding LNPs with diameters of ~300 nm. This approach also allows encapsulation of intact IgG. No chemical or sequence modification of the IgG is required. Treatment of cells with LNPs encapsulating IgGs thus formulated resulted in a remarkable cytosolic distribution of the IgGs, allowing them to interact with the target proteins in the cells and exert the expected pharmacological activities. The approach is promising not only in terms of intracellular IgG delivery, but also in terms of the feasibility of expanding the scope of LNP-based delivery systems developed to date. Future combinations of other IgG delivery approaches developed at the cultured cell level may lead to more sophisticated and efficient delivery systems. This study has demonstrated the impact of using liquid droplets, and thus provides a novel platform for the development of intracellular delivery systems using LNPs.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge at

<https://pubs.acs.org/doi/10.1021/acs.molpharmaceut>

The secondary antibodies used in this study; characterization of LPNs and the encapsulation efficiency of IgG; characterization of [IgG_polyE]LPNs before and after purification; structures of the lipids used to prepare the lipid nanoparticle; and the HiBiT/LgBiT split luciferase (NanoBiT) system for evaluation of cytosolic IgG translocation (PDF)

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Author Contributions

Y.H. designed and executed the experiments with Y.K. and C.K. H.H. provided advice for experimental design. S.F. directed the project and analyzed the data and wrote the paper with Y.H., Y.K., and H.H.

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Notes

§ The total lipid (L_{total}) of vesicles is considered to be proportional to their surface area.

Assuming that a total of n vesicles with radius r are formed from the same amount of total lipid L_{total} in each preparation, the following relationship can be formed:

$L_{\text{total}} = n \times 4\pi r^2 A$ (A is an appropriate coefficient to convert the vesicle surface area to the amount of lipid).

The net vesicle volume (V_{total}) is approximated as:

$$\begin{aligned} V_{\text{total}} &= n \times (4/3)\pi r^3 \\ &= n \times 4\pi r^2 \times r/3 \\ &= L_{\text{total}}/A \times r/3 \\ &= L_{\text{total}}/3A \times r \end{aligned}$$

Using the same amount of lipid, L_{total} is constant and V_{total} is proportional to r . Therefore, the formation of larger diameter vesicles results in the generation of larger net luminal volumes encapsulated in vesicles.

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