

Polysaccharide-based coacervate microgel bearing cationic peptides that achieve dynamic cell-membrane structure alteration and facile cytosolic infusion of IgGs

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Abstract

Conjugates of the biocompatible polysaccharide pullulan with a cell membrane permeabilizing peptide L17E (PL-L17Es) were prepared with the aim of producing complex coacervates with pronounced intracellular antibody (IgG) delivery activity and stable structures. Coacervates with diameters of a few μm were formed simply by mixing PL-L17Es with IgG labeled with negatively charged fluorescent moieties of Alexa Fluor 488 [IgG(AF488)]. The coacervate resulted in a pronounced cytosolic infusion of IgG(AF488) and IgG binding to the target proteins inside the cell. The droplet structures were maintained even under high salt conditions and the fluorescence in the droplet was not recovered after photobleaching, suggesting the formation of complex coacervate microgels. Dynamic changes in cell membrane structure to entrap the coacervate microgels were captured by confocal and electron microscopy, resulting in cytosolic IgG infusion. The use of M-lycotoxin instead of L17E resulted in a coacervate microgel with marked IgG delivery activity even in the presence of serum. Successful IgG delivery to primary hepatocytes, undifferentiated induced pluripotent stem (iPS) cells, and iPS cell-derived intestinal epithelial cells was also achieved. The construction of complex coacervate microgels with design flexibility and the validity of intracellular IgG delivery with high salt stability were thus demonstrated.

Keywords

peptides, antibodies, coacervate, pullulan, intracellular delivery

Introduction

Coacervates are typically formed by the self-assembly of two oppositely charged macromolecules via liquid-liquid phase separation (LLPS).¹⁻² This feature allows easy formulation of therapeutic molecules with different physicochemical properties in a non-covalent manner, simply by mixing with other materials to form complexes. The ease of formulation can be an advantage of coacervates over other delivery vehicles. The possible design and development of systems that efficiently yield coacervates and release therapeutic molecules at a proper timing in target cells has also attracted research attention.³⁻⁴ Coacervates have also been used to aid in encapsulation of therapeutic biomacromolecules (e.g., siRNA and mRNA) into other vehicles such as lipid- and polymer-based nanoparticles.⁵⁻⁶ Therefore, there is growing research interest in coacervates as a means of intracellular delivery of biomacromolecules with therapeutic potential.⁷⁻¹⁰ Although coacervates hold a great promise as intracellular delivery vehicles or platforms, an inherent weakness would be their instability under conditions of high salt concentration or high dilution, which may become an obstacle to their practical application.

Numerous approaches have been reported for the delivery of nucleic acids, including siRNA and mRNA, using coacervates and other delivery vehicles such as lipid- and polymer-based nanoparticles.¹¹⁻¹² Compared to nucleic acid delivery approaches, far fewer approaches have been reported for the delivery of proteins into cells, with intracellular delivery of antibodies being one of the challenges. Antibodies, especially immunoglobulin G (IgG), are the ultimate molecularly targeted drugs.¹³ Taking advantage of their high target recognition ability, a large number of antibody-based drugs have been developed for clinical use as representative protein-based biopharmaceuticals. Due to their inability to cross biological membranes, the vast majority of targets for antibody therapeutics are currently located outside of cells or on the cell membrane (plasma membrane).¹⁴⁻¹⁹ The development of methods to deliver antibodies into cells to bind target molecules inside the cell should open up new therapeutic applications. Not only for clinical trials, but also for basic cell biology studies, such methods should have a significant impact on the understanding of the localization of the molecules of interest and the modes of molecular interaction in cells. Although approaches have been developed to deliver antibodies into cells in the presence of

membrane permeabilizing peptides,^{20,21-29,30-31} by conjugation or complex formation with cell-penetrating peptides,³²⁻³³ or by encapsulation in lipid, polymer, and other nanoparticles,³⁴⁻³⁹ there is still room to improve the efficiency of intracellular delivery and to establish formulations to deliver antibodies together with membrane permeabilizing molecules to the target organs or cells. Therefore, new concepts for high efficiency antibody delivery into cells have been awaited.

L17E is a potent antibody (IgG) delivery peptide developed by our research group, which has an amphiphilic property rich in cationic and hydrophobic amino acids. L17E transiently permeabilizes the cell membrane (plasma membrane) and allows the cytosolic translocation of extracellular IgGs.²⁰ We designed a trimer of L17E tagged with an Fc-binding peptide [FcB(L17E)₃] to improve the delivery efficiency.⁴⁰ Interestingly and surprisingly, FcB(L17E)₃ formed coacervates or liquid droplets with IgGs decorated with the negatively charged fluorophore Alexa Fluor 488 [IgG(AF488)]. A marked intracellular infusion of IgGs from the coacervate was achieved by attachment to cells, which achieved immediate cytosolic IgG distribution within one minute. Cytosolic translocation of IgG from the coacervate was achieved through the plasma membrane or at a very early stage of endocytosis, accompanied by a dynamic change in the plasma membrane structure, suggesting the presence of a unique mode of cellular uptake. Sun and coworkers also reported coacervate-mediated intracellular delivery of IgG and other proteins using a histidine-rich peptide with pH- and redox-sensitive modification.⁴¹ Since instability of coacervates can be an obstacle to their therapeutic application, stabilizing coacervate structures while retaining the above effective intracellular IgG delivery activity would lead to the establishment of a novel and useful design platform for intracellular delivery and cell manipulation. Increasing the interaction between L17E and IgGs may result in a more stabilized complex coacervate compared to those made from FcB(L17E)₃ and IgG(AF488), which may be more amenable to therapeutic application if the coacervate retains the ability to efficiently deliver IgGs intracellularly. In addition, one of the potential problems in designing intracellular delivery systems for biomacromolecules is the reduction in delivery efficiency in the presence of serum.⁴² Since many previous reports on droplet-based intracellular delivery have not explicitly mentioned the effect of serum on

delivery,^{9, 41, 43} it is preferable that our new systems function in serum-containing media, as was the case with the FcB(L17E)₃/IgG(AF488) coacervate-based IgG delivery system.

With this in mind, we prepared the conjugates of the polysaccharide pullulan with membrane permeabilizing peptides such as L17E and analyzed their applicability for coacervate formation with IgGs and for intracellular delivery of IgGs (Figure 1A). The use of conjugates of pullulan with multiple numbers of L17E segments (~66 molecules of L17E were conjugated to a pullulan chain of ~100 kDa) resulted in the formation of coacervate droplets with diameters of ~2 μm when mixed with IgG(AF488). The droplets formed by pullulan L17E conjugates (PL-L17E) with IgG(AF488) had much higher stability under high salt conditions and lower mobility of IgG(AF488) in the droplets compared to those in FcB(L17E)₃/IgG(AF488) liquid droplets, presumably due to increased entanglement between L17E and IgG(AF488) within the droplets, successfully forming complex coacervate microgels. Importantly, the complex coacervate microgels retained the ability to rapidly deliver IgGs to the cytosol as in FcB(L17E)₃/IgG(AF488) coacervate droplets. Although a significant reduction in IgG delivery activity was observed for the PL-L17E/IgG(AF488) coacervate microgel in the presence of serum, the use of a more membrane-lytic peptide, M-lycotoxin (MLTX),⁴⁴ in place of L17E resulted in marked IgG delivery activity. Thus, the complex coacervate microgel delivery system retains both stability and high delivery activity, overcoming previous obstacles to the development of intracellular delivery systems using coacervates.

Here we describe the preparation of the conjugates of membrane lytic peptides with pullulan, coacervate droplet formation with IgG(AF488), marked cytosolic infusion of IgG(AF488), characterization of the complex coacervates as microgels, and evaluation of IgG delivery activity under serum-containing conditions and to primary hepatocytes, undifferentiated induced pluripotent stem (iPS) cells, and iPS-cell derived intestinal epithelial cells were described.

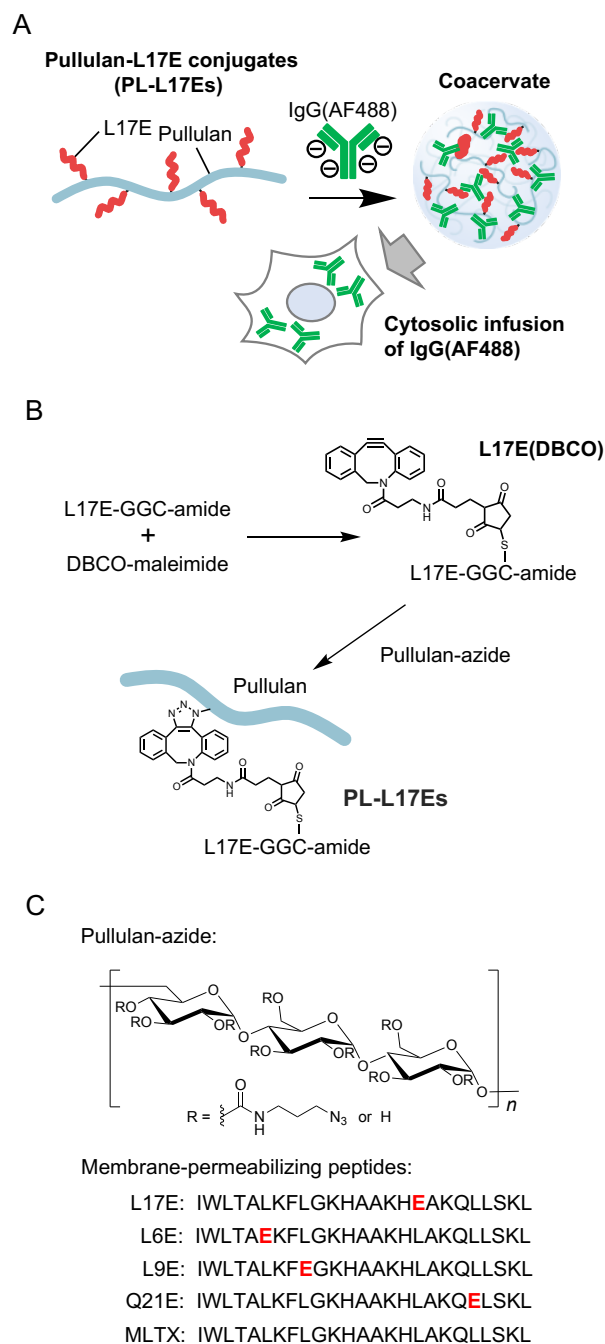


Figure 1. (A) Schematic representation of cytosolic IgG infusion from coacervate microgel. (B) Preparation of pullulan-L17E conjugates (PL-L17Es). Coacervate microgels, which achieve a marked cytosolic delivery of IgGs decorated with the negatively charged fluorophore Alexa Fluor 488 [IgG(AF488)], were typically formed by mixing with pullulan (~100 kDa consisting of ~550 glucose units) conjugated with ~66 molecules of L17E (PL-L17E12/100). IgGs modified with 7~9 molecules of Alexa Fluor 488 were employed. (C) Structures of pullulan azide and membrane permeabilizing peptides used in this study. L17E, L6E, L9E, and Q21E peptides were derived from a spider toxin M-lycotoxin (MLTX). Glutamate substitution (shown in red) was performed to modulate the lytic activity.²⁰

Results and Discussion

Design and preparation of pullulan-L17E conjugates (PL-L17Es). Pullulan is a polysaccharide polymer of high biocompatibility, consisting of three glucose units linked by an α -1,4 glycosidic linkage, and these three glucose units are linked by an α -1,6 glycosidic linkage. Akiyoshi and coworkers have shown that cholesterol-modified polysaccharide pullulan (CHP) forms a self-assembled polysaccharide nanogel, where hydrophobic interaction between cholesterol moieties works as physical cross-linking to allow nanoparticle formation.⁴⁵⁻⁴⁶ The applicability of CHP as carriers of proteins and other bioactive materials for clinical cancer immunotherapy and nasal vaccine has also been demonstrated.⁴⁷⁻⁴⁸ We envisioned that modification of pullulan with L17E might lead to formation of self-assembled particles or coacervates bearing IgGs that may be potentially applicable to in vivo IgG delivery. Since L17E is rich in both cationic and hydrophobic amino acid residues, IgGs were modified with Alexa Fluor 488 [IgG(AF488)], bearing two sulfonic acid moieties, to enhance the interaction with L17E and to visualize cellular uptake. The importance of IgG modification with negative charges on coacervate droplet formation and intracellular IgG influx was also shown in our previous study.⁴⁰

For the preparation of pullulan-L17E conjugates, pullulan-azide was prepared using pullulan (consisting of $\sim$ 550 glucose units; $\sim$ 100 kDa) according to the reported procedure.⁴⁹ This resulted in pullulan-azide with 12.6 azide units per 100 glucose units (Figure S1). For conjugation to pullulan-azide, L17E bearing Gly-Gly-Cys-amide (L17E-GGC-amide) was prepared, which was modified with dibenzocyclooctyne (DBCO)-maleimide to yield L17E(DBCO), allowing copper-free click reaction with pullulan-azide (Figure 1B, C).

To investigate the significance of the modification ratio of L17E, five different sets of pullulan conjugated with L17E (PL-L17Es: PL-L17E_{1/100}, PL-L17E_{4/100}, PL-L17E_{8/100}, PL-L17E_{12/100}, PL-L17E_{14/100}) were prepared. The number after PL-L17Es represents the equivalents of L17E to 100 monosaccharide units of pullulan subjected to the click reaction. The actual modification ratios determined by ¹H NMR for PL-L17E_{1/100}, PL-L17E_{4/100}, PL-L17E_{8/100}, PL-L17E_{12/100}, and PL-L17E_{14/100} were 0.66, 3.53, 7.39, 11.41, and 12.21, respectively (Figure S2), indicating that the click reaction proceeded almost quantitatively. Note that for the preparation of PL-L17E_{14/100}, an excess amount of L17E was used over the

azide moiety (12.6 moieties per 100 glucose units), and thus the conjugate yielded only slightly higher L17E modification ratios than PL-L17E_{12/100}.

Particle-like structure formation of IgG(AF488) with PL-L17Es achieving facile cytosolic IgG(AF488) infusion. PL-L17Es and IgG(AF488) were mixed to give a molar ratio of L17E:IgG (8:1) (L17E concentration, ~40 μ M), incubated at 23°C for 10 min, and then diluted to give a final L17E concentration of 3 μ M. Confocal laser scanning microscopy (CLSM) analysis showed that all mixtures except the one containing PL-L17E_{1/100} and IgG(AF488) formed particles of ~1-3 μ m diameter (Figure 2A, differential interference contrast (DIC) images). The inclusion of IgG(AF488) in these particles was suggested by the fact that these particles showed signals of AF488. Dynamic light scattering (DLS) particle size distribution also showed the particle size distribution of 1-4 μ m with a median of 2 μ m by incubation of PL-L17E_{12/100} with IgG(AF488) (Figure S3). No significant particle formation of these sizes was observed using non-AF488 labeled IgG or in the absence of IgG as in the case of FcB(L17E)₃/IgG(AF488).⁴⁰

We next examined whether these particles had the ability to deliver IgGs into the interior of cells (Figure 2B). HeLa cells were treated with particles formed from IgG(AF488) with PL-L17E_{4/100}, PL-L17E_{8/100}, PL-L17E_{12/100}, and PL-L17E_{14/100} for 1 h. PL-L17E_{1/100} did not form particles and was therefore not used in the evaluations. The WST-8 assay, which assesses mitochondrial metabolic activity, was then performed on the PL-L17Es to estimate the concentration at which cellular treatment would not be associated with significant cytotoxicity (Figure S4). No significant cytotoxicity was observed at 3 μ M (as L17E concentration) for PL-L17E_{12/100} and PL-L17E_{14/100}. Therefore, cells were incubated with these particles prepared as in Figure 2A for 1 h. Strong IgG(AF488) signals were observed in ~70% of the cells, distributed throughout the cells (Figure 2C), suggesting a cytosolic influx of IgG(AF488) from the particles. The efficacy was comparable or slightly higher than that obtained with FcB(L17E)₃/IgG(AF488) coacervate droplet.⁴⁰ Since some cytotoxicity was observed in the cases of PL-L17E_{4/100} and PL-L17E_{8/100} at 3 μ M (as L17E concentration), the cells were treated with the particles prepared to give a final L17E concentration of 1 μ M. A lower degree of cytosolic distribution of IgG(AF488) signals was observed, both in terms of percentage of cells with signals and fluorescence intensities; the percentage of cells with

cytosolic IgG(AF488) signals was ~ 10 and $\sim 25\%$, respectively (Figure 2B and C). As a control, when cells were treated with $770 \mu\text{g/mL}$ IgG(AF488) in the presence of $40 \mu\text{M}$ L17E (not conjugated to pullulan), $\sim 40\%$ of the cells had IgG(AF488) signals, demonstrating the impact of the present approach using PL-L17E_{12/100} and PL-L17E_{14/100}. Since PL-L17E_{12/100} and PL-L17E_{14/100} gave comparable results, PL-L17E_{12/100} was used for the following study.

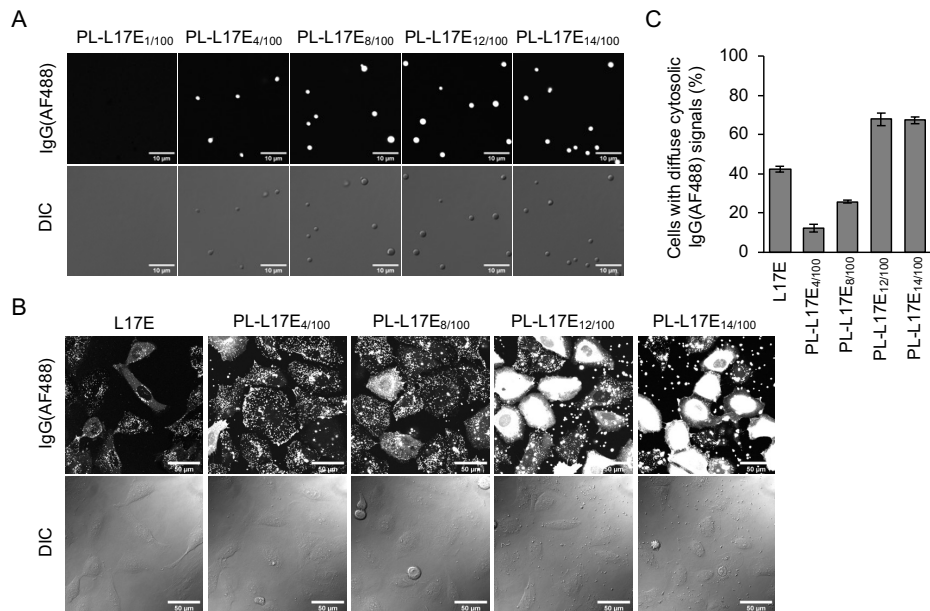


Figure 2. (A) Particle-like complex formation between PL-L17Es and IgG(AF488). Scale bars, 10 μm . (B) Confocal microscopic observation of HeLa cells after incubation with the particles formed by PL-L17Es in the presence of IgG(AF488) for 1 h. Scale bars, 50 μm . (C) Cells with cytosolic diffuse IgG(AF488) signals in (B). Results are presented as mean $\pm$ standard error (SE) (n=4).

Notably, particle-like structure formation is important for PL-L17E_{12/100}-mediated cytosolic IgG delivery, but is not sufficient to ensure cytosolic IgG(AF488) infusion. L17E was designed to attenuate the lytic activity of the cationic amphiphilic lytic peptide M-lycotoxin (MLTX)⁴⁴ by substituting the hydrophobic leucine residue at position 17 in the potential hydrophobic face of MLTX with a negatively charged glutamic acid, where the position of the Glu substitution had a significant effect on the delivery activity.²⁰ Although marked cytosolic IgG influx was achieved in the presence of L17E, considerably lower cytosolic IgG delivery

was achieved in the presence of L6E and L9E peptides – both peptides had exactly the same amino acid composition as L17E, but the positions of the leucine to glutamic acid substitution are different (i.e. at positions 6 and 9) (Figure S5A). Although a mixture of IgG(AF488) with the pullulan conjugates with L6E-GGC-amide and L9E-GGC-amide (PL-L6E_{12/100} and PL-L9E_{12/100}) similarly prepared with PL-L17E_{12/100} formed particle-like structures, very few cells had cytosolic IgG(AF488) signals (Figure S5B).

Particle-like structures formed by PL-L17E_{12/100} and IgG(AF488) were more like gels than liquid droplets. Electrostatic interaction plays an important role in the stabilization of liquid droplets or coacervates, and high salt concentrations generally destabilize droplet structures.⁵⁰ Since turbidity is a measure of liquid droplet formation, the effect of NaCl concentration was analyzed for the particle-like structures formed by PL-L17E_{12/100} and IgG(AF488) (Figure 3A). There was an obvious decrease in absorbance at 650 nm of the solutions containing the PL-L17E_{12/100}/IgG(AF488) particles with increasing NaCl concentration. However, even at 250 mM and 2 M NaCl concentrations, the absorbance at 650 nm was maintained at 77 and 48% of that at 124 mM NaCl concentration, respectively (Figure 3A). This was in marked contrast to the results obtained for the FcB(L17E)₃/IgG(AF488) complex – very few particle structures were retained at NaCl concentrations greater than 200 mM.⁴⁰ The significant retention of the particle-like structures at 2 M NaCl concentration was also confirmed by CLSM observation (Figure 3B). We then performed fluorescence recovery after photobleaching (FRAP) analysis on the particle-like structures. FRAP is often used to determine the molecular dynamics and mobility of fluorescent components in the phase-separated liquid droplets.²⁵ Although fluorescence recovery in liquid droplets is generally achieved in a few minutes or less, no significant recovery was obtained even after 30 min post-photobleaching (Figure 3C). Similar trends were observed for PL-L17E_{4/100}, PL-L17E_{8/100}, and PL-L17E_{14/100} (Figure S6A).

It would be useful to know the percentage of PL-L17E and IgG(AF488) captured in the particle-like structures. This was evaluated by the concentration of L17E and IgG(AF488) remaining in the supernatant after centrifugation of the particles. It was found that ~50% of the added PL-L17E_{12/100} and ~35% of the IgG(AF488) were used for particle formation (Figure S6B). Similar trends were observed for the PL-L17E_{8/100}/IgG(AF488) and PL-

L17E_{14/100}/IgG(AF488) complexes. The lesser extent of entrapment of PL-L17E_{4/100} and IgG(AF488) was observed in the case of the PL-L17E_{4/100}/IgG(AF488) complex compared to other complexes.

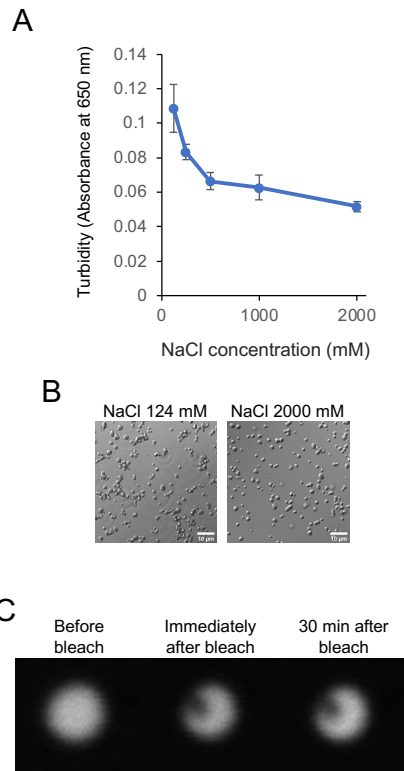


Figure 3. (A) Effect of NaCl concentration on the stability of the particle-like complex of PL-L17Es with IgG(AF488). Results are presented as mean $\pm$ SE (n=3). (B) DIC images of the samples at 124 and 2000 mM NaCl concentration. (C) FRAP analysis of IgG(AF488) in the particle-like complex of PL-L17E_{12/100} with IgG(AF488).

Involvement of dynamic cell membrane restructuring and a unique mode of cellular uptake leading to cytosolic IgG(AF488) infusion: Similarities and differences with cytosolic IgG(AF488) infusion using FcB(L17E)₃. In FcB(L17E)₃/IgG(AF488) complex-mediated cytosolic IgG(AF488) delivery, attachment of the liquid droplet-like FcB(L17E)₃/IgG(AF488) complex to the cell membrane was observed to result in immediate cytosolic influx of IgG(AF488);⁴⁰ this process was accompanied by reorganization of cell membrane and F-actin structures and was not observed at 4°C, indicating the necessity of

cellular machinery to achieve the influx and ruling out the possibility of influx due to simple pore/fracture formation in the cell membrane (plasma membrane). We therefore investigated whether the similar modes of IgG(AF488) delivery were carried out in PL-L17E_{12/100}-mediated delivery and then performed time-lapse observation.

After addition of the PL-L17E_{12/100}/IgG(AF488) complex to the cell culture medium, it usually took 20-30 min for the particle structures to sediment on the cells (e.g., the particle in Figure 4A, 28 min, indicated by the arrowhead), which was accompanied by a cytosolic influx of IgG(AF488) signals throughout the cell (Figure 4A, 31-37 min, arrowhead). The time required for the complete distribution of IgG(AF488) signals throughout the cell was in the range of several minutes, which is slightly longer than that observed for the FcB(L17E)₃/IgG(AF488) complex (~1 min). The dynamic structural change of the cell membrane associated with the reorganization of the cytoplasmic F-actin cytoskeleton was accompanied by the influx of IgG(AF488) from the FcB(L17E)₃/IgG(AF488) complex. Similarly, the cell membrane was recruited around the particle to wrap the particle prior to IgG(AF488) influx, as shown in the enlarged differential interference contrast (DIC) images in Figure 4A, lower panels (see also Supporting Information Video S1 and S2). Presumably because the PL-L17E_{12/100}/IgG(AF488) complex has a more gel-like structure, the particle structures interacting with cell membranes were captured by scanning electron microscopy (SEM) (Figure 4B(i)). In addition to the particle-like structures that were clearly observed on the cell membrane (yellow arrowheads), those whose contours were less evident against the cell membrane were also observed (white arrowheads). Notably, the higher resolution image of the particle-like structure marked with a yellow arrowhead in the boxed area in Figure 4B(i) showed that fine membrane protrusions were attached around the particle (Figure 4B(ii), yellow arrowhead; see also Figure S7, middle panels). However, in the case of the particle-like structure indicated by the white arrowhead, the seamless membrane structure was observed to cover the particle-like structure, as if the particle-like structure was taken up into the cells via a specific cellular uptake system and the cell membrane structure was restored (Figure 4B(ii), white arrowhead).

The next question is the intracellular fate of the internalized particle-like structures. To obtain information, the above uptake experiment was performed using HeLa cells expressing

Lyn11-mCherry (Figure 4C). Lyn is a member of the Src family of kinases that is anchored to the inner leaflet of the cell membrane by myristoylation.⁵¹ Lyn11 is the responsible sequence (GCIKSKGKDSA) for myristoylation and thus Lyn11-mCherry can be used for cell membrane labeling.⁵² Remarkably, the AF488 signals in the z-stacks together with the line in Figure 4C(i) suggested the presence of particle-like structures of μm size within the cells (Figure 4C(ii), AF488). The signals of mCherry were observed surrounding the AF488 signals, suggesting that the particle structures were trapped in large endosome-like vesicular compartments (Figure 4C(ii), mCherry). These results strongly suggest that the particles were wrapped by the protrusions of the cell membrane and engulfed into the cells while retaining the particle structures. This mode of uptake was reminiscent of our recent observation of cellular uptake of 3 μm plastic beads by HeLa cells, where the beads were captured by membrane protrusions and delivered into cells encapsulated by vesicular compartments.⁵³ The modes of uptake of PL-L17E_{12/100}/IgG(AF488) particles or 3 μm plastic beads may have some similarity to phagocytosis. However, it is important to note that HeLa cells are not phagocytic cells and would not be expected to endocytose such a particle of a few μm in diameter. This suggests the possible involvement of non-canonical or atypical modes of cellular uptake for the PL-L17E_{12/100}/IgG(AF488) particles. Transient membrane permeabilization due to the action of L17E may occur during the uptake without significant cellular damage. In addition, the above modes of IgG(AF488) infusion by L17E_{12/100}/IgG(AF488) particles have some similarities with those of FcB(L17E)₃/IgG(AF488) complex, but there were also distinct differences; Although similar types of remodeling of cell membrane structures were induced by cellular interaction with FcB(L17E)₃/IgG(AF488) complexes, cytosolic IgG(AF488) influx occurred immediately at the cell surface or at a very early stage of endocytosis. No particle-like structure retained in the cytoplasm was observed, presumably because of differences in the rigidity of the complex structures.

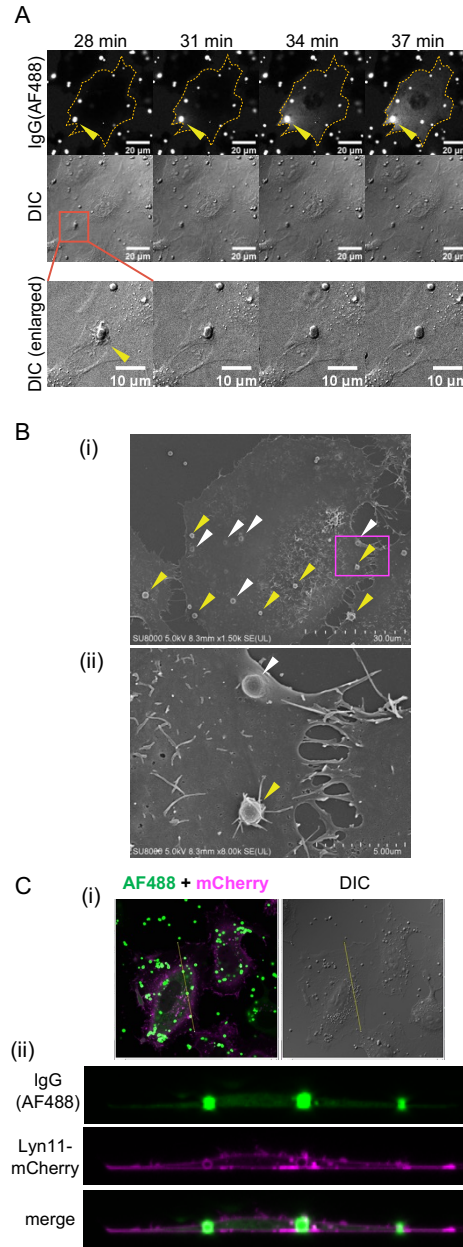


Figure 4. (A) Time-lapse imaging of cells treated with a mixture of PL-L17E_{12/100} and IgG(AF488). Arrowheads indicate a particle-like structure that interacts with the periphery of a cell, resulting in the distribution of IgG(AF488) signals within the cells (the cell boundary is highlighted with a dotted line). The enlarged DIC images corresponding to the position indicated by the red square are shown at the bottom. Note that the membrane structures surrounding the particle (28 min, arrowhead) became less visible with the infusion of IgG(AF488) into the cells. Scale bars, 20 μm (top and middle images) and 10 μm (bottom images) (B) (i) SEM analysis (×1,500) of cells treated with the PL-L17E_{12/100}/IgG(AF488) complex. Yellow arrowheads represent the typical particle-like structures on the cell membranes with fine membrane protrusions attached around the particles. White arrowheads represent the particle-like structures covered by smooth membranes. Scale bar, 30 μm. (ii) Magnified image (×8,000) of the area highlighted by the magenta box in (i). Scale bar, 5 μm. (C) (i) Confocal microscopy image of Lyn11-mCherry-expressing HeLa cells treated

with the L17E_{12/100}/IgG(AF488) complex. (ii) Z-stack image of the HeLa cells along the line in (i).

The involvement of F-actin in the above process was further confirmed by the inhibition of IgG(AF488) infusion in the presence of cytochalasin D (CytD) (Figure S8A). CytD binds to F-actin and inhibits actin reorganization. A significant decrease in the number of cells with cytosolic IgG(AF488) signals (~65% to ~15% of total cells, Figure S8B) was observed in the presence of CytD, suggesting the involvement of F-actin reorganization in the infusion process. The importance of cholesterol for cellular uptake with dynamic membrane structural changes was suggested by methyl- β -cyclodextrin (M β CD) treatment, which reduced the number of cells with cytosolic IgG(AF488) signals to ~35% (Figure S8A and B).

The IgG(AF488) infusion via complex formation with PL-L17E_{12/100} was also achieved in cell lines other than HeLa (human cervical cancer derived), and these were: HT1080 (human sarcoma-derived), T24 (human urinary bladder carcinoma-derived), A431 (human epidermoid carcinoma-derived), Raw264.7 (murine tumor macrophage-derived), and HEK-293T (human renal epithelial-like cell-derived) (Figure S9). Strong cytosolic IgG(AF488) signals were observed in these cells, suggesting the applicability of this liquid droplet-mediated IgG delivery to different cell types.

Interestingly, although PL-L9E_{12/100}/IgG(AF488) did not result in marked cytosolic IgG(AF488) distribution (Figure S5), similar modes of entrapment of particles formed from PL-L9E_{12/100}/IgG(AF488) by membrane protrusions and the possible traces of entrapment of the particles into cells were observed on cell surfaces (Figure S7, lower panels). This suggests that the deficiency of IgG(AF488) in cytosolic infusion of PL-L9E_{12/100}/IgG(AF488) may not be due to the difficulty of endocytic uptake, but to the rupture of the membranes of endosome-like vesicular compartments.

Use of peptides with stronger membrane interaction for IgG(AF488) delivery in serum containing conditions. The above results have demonstrated the promise of PL-L17E_{12/100} as a platform for intracellular IgG delivery, which was studied under serum-free conditions to directly understand the cellular response to PL-L17Es and the basic delivery capability without considering the effect of serum. On the other hand, a significant decrease in IgG delivery efficacy was observed using PL-L17E_{12/100} in the presence of serum. While

about two thirds of the cells exhibited diffuse IgG(AF488) signals upon treatment with PL-L17E_{12/100}/IgG(AF488) in serum-free condition, only ~20% of the cells exhibited the signals in the presence of serum (Figure 5B (i) and (ii), PL-L17E_{12/100}). Therefore, peptides with stronger membrane interacting abilities were used to be labeled with pullulan instead of L17E to increase the ability of cytosolic IgG(AF488) infusion – the membrane perturbing abilities of membrane interacting peptides are often diminished in the presence of serum⁴² Q21E peptide or M-lycotoxin bearing GGC linker at their C-terminus (Figure 5A) were similarly tagged with pullulan as in Figure 1B (PL-Q21E_{12/100} and PL-MLTX_{12/100}, respectively) and their IgG(AF488) infusion ability was evaluated – these peptides had stronger membrane perturbation ability than L17E.²⁰

More pronounced cytosolic IgG(AF488) infusion was achieved by treating cells with the IgG(AF488) complexes formed with PL-Q21E_{12/100} and PL-MLTX_{12/100} in the absence of serum (85% and 92%, respectively; Figure 5B(i)). Notably, even in the presence of serum, PL-Q21E_{12/100} and PL-MLTX_{12/100} yielded a higher number of cells with cytosolic IgG(AF488) (41% and 82%, respectively; Figure 5B(ii)). Q21E and MLTX *per se* had considerable cytotoxicity (the concentration to yield 50% cell death: 3.54 and 1.36 μ M, respectively) and were not ideal for use to promote membrane permeabilization to allow translocation of IgGs into cells, and thus L17E was used for this purpose.²⁰ However, when labeled with pullulan, no marked cytotoxicity was observed for PL-Q21E_{12/100} and PL-MLTX_{12/100} under the conditions used (peptide concentration, 3 μ M) (Figure S10). Similar to PL-L17E_{12/100}/IgG(AF488) in serum-free condition (Figure 4A), a significant cytosolic IgG(AF488) uptake was observed by complex formation with PL-MLTX_{12/100} even in serum-containing medium (Figure S11 and Supporting Information Video 3). Since PL-MLTX_{12/100} had the most potent cytosolic IgG(AF488) infusion activity in the presence of serum, PL-MLTX_{12/100} was used in the following studies to confirm the validity of the infusion system.

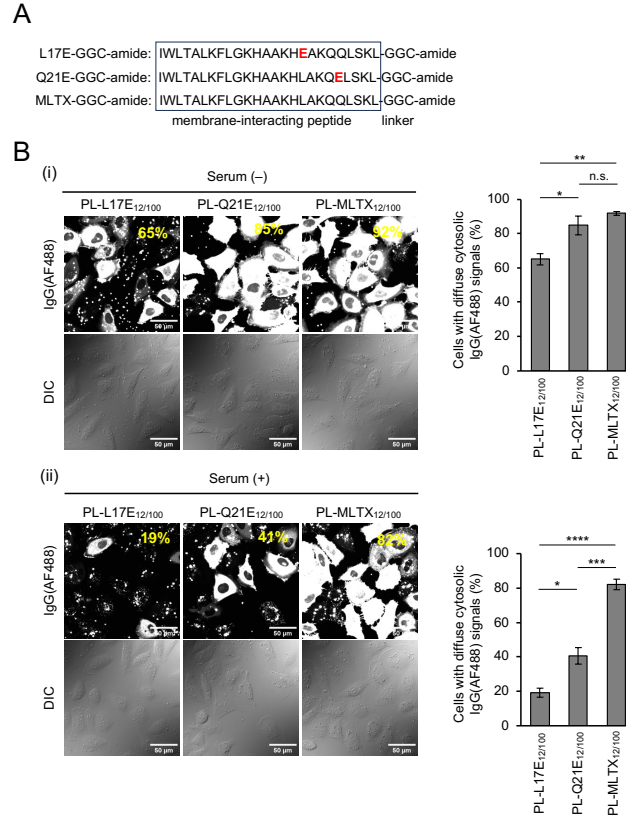


Figure 5. (A) The sequences of L17E-GGC-amide, Q21E-GGC-amide, and MLTX-GGC-amide. (B) Distribution of IgG(AF488) signals in the cells treated with the IgG(AF488) complexes formed with PL-L17E_{12/100}, PL-Q21E_{12/100}, and PL-MLTX_{12/100} in the absence (i) and presence (ii) of serum at 37°C for 1 h. Scale bars, 50 μ m. The percentages of cells with diffuse cytosolic IgG(AF488) signals are given on the IgG(AF488) images. Results are presented as mean $\pm$ SE (n=3). *, p<0.05, **, p<0.01, ***, p<0.001, ****, p<0.0001, n.s.; not significantly different by Tukey-Kramer's honestly significant difference test.

Target recognition activity of delivered IgG. To confirm that delivered IgGs still retain their ability to bind their targets, AF488-labeled mouse anti-nucleic pore complex IgG [anti-NPC(AF488), 6.6 dye molecules per IgG molecule] was used as a model IgG for delivery using PL-MLTX_{12/100}. Treatment of cells with the PL-MLTX_{12/100}/anti-NPC(AF488) complex under serum-containing conditions resulted in intense fluorescence signals around the nucleus

(Figure 6, arrowheads). Of course, the partial degradation or denaturation of the IgGs should be accompanied by the coacervate-mediated intracellular delivery, but the above results strongly suggest that a certain fraction of the IgGs thus delivered into cells retain the intrinsic binding ability to the target proteins in the cells, supporting the validity of this approach. The PL-MLTX_{12/100}/anti-NPC(AF488) or PL-L17E_{12/100}/anti-NPC(AF488) complexes resulted in a comparable level of accumulation of anti-NPC(AF488) signals around the nuclear membrane in the absence of serum (Figure S12A and B).

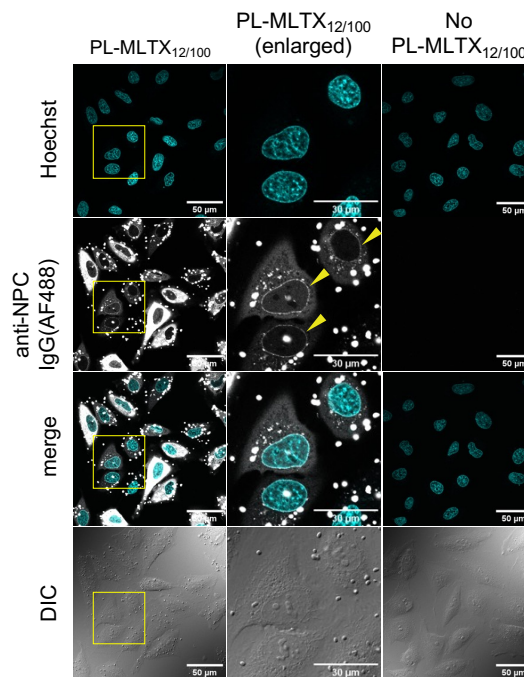


Figure 6. (A) Nuclear membrane accumulation of anti-nuclear pore complex (NPC) IgG(AF488) from the PL-MLTX_{12/100}/anti-NPC-IgG(AF488) complex after incubation in serum-containing α -MEM for 1 h. Concentrations of PL-MLTX_{12/100} and anti-NPC-IgG(AF488) used for complex formation: 3 μ M (as concentration of MLTX) and 56 μ g/mL, respectively.

Cytosolic infusion of IgG(AF488) to primary hepatocytes and induced pluripotent stem (iPS) cells. The feasibility of intracellular delivery and targeting of IgGs using coacervate microgels formed with pullulan tagged with membrane permeabilizing peptides, including L17E and MLTX, was thus exemplified, with the involvement of dynamic structural changes of the cell membrane accompanied by dynamic actin rearrangement playing a critical

role. The potential applicability of the delivery concept to different cell lines was exemplified as shown in Figure S9. While further elaboration, especially to reduce the particle size of the coacervate microgels (a few μm), is expected for in vivo application, especially when considering intravenous administration, we next investigated whether IgG infusion to primary cells was achievable as a first step in this direction. The liver is the most accessible organ for drugs in the bloodstream. The liver is composed of various cell types, including hepatocytes, macrophages (Kupffer cells) and stellate cells, and hepatocytes are the major drug targets in the liver.⁵⁴ Therefore, the ability of the PL-MLTX_{12/100}-based coacervate microgel to deliver IgG(AF488) to primary hepatocytes was investigated.

Isolated primary hepatocytes from the liver of C57BL/6 mice were treated with the PL-MLTX_{12/100}/IgG(AF488) microgel or with IgG(AF488) for 1 h (Figure 7). While a marked cytosolic signal of IgG(AF488) was observed in most of the cells for the cells treated with the PL-MLTX_{12/100}/IgG(AF488) microgel, no significant cytosolic IgG(AF488) signals were observed for the cells treated with IgG(AF488) without forming the microgel with MLTX_{12/100}, indicating the potential feasibility of PL-MLTX_{12/100} as an IgG delivery platform in the liver or in vivo. The Z-stack cross section image along with the line in the image of PL-MLTX_{12/100} and IgG(AF488) suggest similar methods of cellular uptake of the coacervate microgel as observed in Figure 4C.

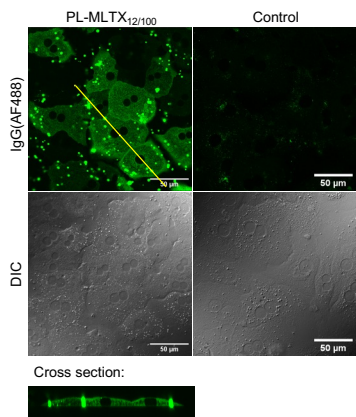


Figure 7. Primary hepatocytes treated with the PL-MLTX_{12/100}/IgG(AF488) complexes or IgG(AF488) alone (control) in serum-free Williams' Medium E for 1 h. Concentrations of PL-MLTX_{12/100} and IgG(AF488) used for complex formation: 3 μM (as concentration of MLTX) and

56 µg/mL, respectively. The Z-stack cross section image along with the line in the image of PL-MLTX_{12/100} and IgG(AF488) is also shown. Scale bars, 50 µm.

The applicability of the PL-MLTX_{12/100}/IgG(AF488) coacervate microgel for IgG delivery to induced pluripotent stem (iPS) cells has also been evaluated. It has been demonstrated that somatic cells and organoids derived from human iPS cells can make a significant contribution to drug discovery for various purposes, including elucidation of disease mechanisms, drug seed discovery, evaluation of drug efficacy and prediction of toxicity.⁵⁵⁻⁵⁶ The modes of cellular uptake in iPS cells may be different from differentiated cells, and it would be noteworthy to confirm that the intracellular delivery to these cells is possible using our coacervate-mediated infusion system. The ability of transient IgG infusion into undifferentiated iPS cells should pave the way for controlling molecular interactions in cells, providing approaches to study the fundamental questions of cell differentiation and to stimulate cell differentiation in specific directions. If successful and efficient cytosolic IgG delivery can be achieved in iPS cell-derived somatic cells, the approach should be beneficial for drug validation.

Undifferentiated iPS cells were treated with the PL-MLTX_{12/100}/IgG(AF488) coacervate microgel for 1 h and IgG(AF488) infusion was evaluated by CLSM (Figure 8A). Although the efficacy may not be satisfactory enough, a significant cytosolic appearance of IgG(AF488) signals was observed predominantly in the cells at the periphery of the colony. On the other hand, no significant cytosolic IgG(AF488) signals were observed in the absence of PL-MLTX_{12/100}. Similarly, significant cytosolic IgG(AF488) infusion was observed from the PL-MLTX_{12/100}/IgG(AF488) coacervate microgel to the intestinal epithelial cells differentiated from iPS cells (Figure 8B). Although there is considerable room to improve the efficacy of IgG infusion in these undifferentiated and differentiated iPS cells at the current stage, the data suggested the possible applicability of the system for IgG delivery to modulate cellular interactions and the eventual cellular events through the future elaboration of the coacervate microgel systems.

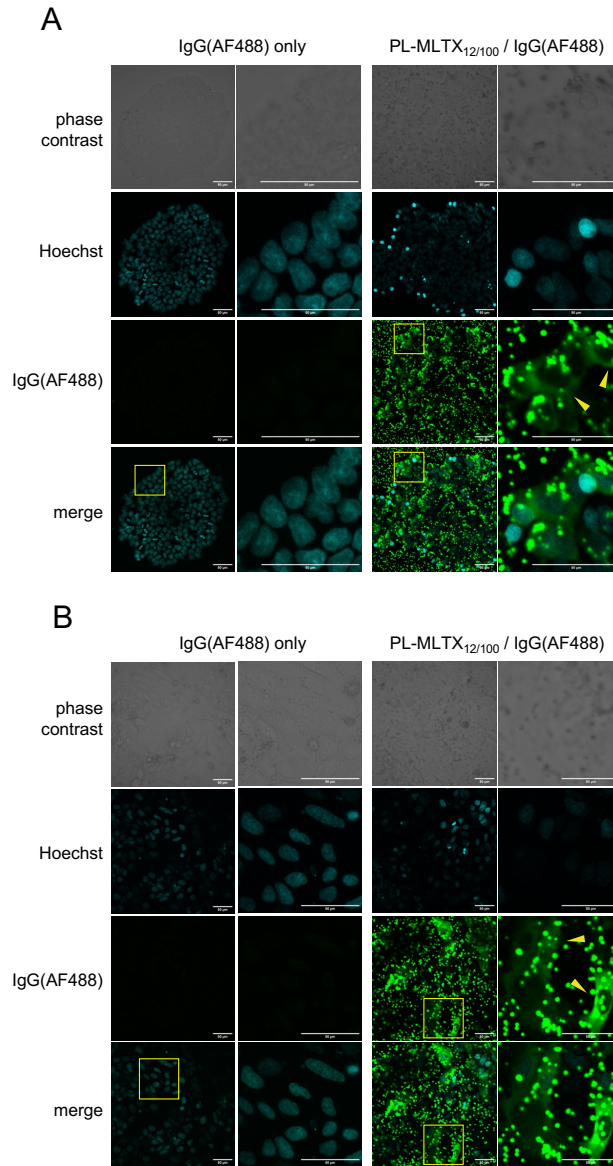


Figure 8. Treatment of (A) undifferentiated iPS cells and (B) iPS cell-derived intestinal epithelial cells with IgG(AF488) and PL-MLTX_{12/100}/IgG(AF488) complexes for 1 h. Enlarged images of the cells corresponding to the boxed area in the left panels are shown in the right panels. Arrowheads indicate the cells with cytosolic IgG(AF488) signals. Since the nuclear pore generally does not allow for translocation of macromolecules of $> \sim 30$ kDa,⁵⁷ this indicates that the majority of IgG(AF488) was delivered to the cytosol while being modified with IgGs, thus retaining IgG structures without suffering from proteolytic degradation. Due to the difficulty of culturing undifferentiated iPS cells on glass dishes and the tendency of these cells tend to form stacked colonies, the images were taken from the cells on plastic dishes, which may make it difficult to obtain high-resolution images. Scale bars, 50 μ m.

Conclusion

We previously reported that by mixing the trimer of L17E tagged with an Fc-binding peptide [FcB(L17E)₃] with IgG(AF488), particle-like droplets were formed by liquid-liquid phase separation, leading to spontaneous infusion and cytosolic distribution of IgGs.⁴⁰ Although the system showed a marked efficacy in IgG infusion, the droplet formation and IgG infusion became less effective at the relatively high NaCl concentration (>200 mM). To obtain more stabilized coacervate structures, we prepared the conjugates of pullulan with L17E (PL-L17Es). We employed pullulan as a model polymer because of its high biocompatibility and the methodological accessibility of conjugate formation reported previously,⁴⁵⁻⁴⁶ although other polymers could be used for the conjugate formation. Coacervate formation was confirmed when PL-L17Es were mixed with IgG(AF488), resulting in the successful delivery of IgG(AF488) into cells. Among the PL-L17Es, one with ~12 molecules of L17E per 100 glucose units (PL-L17E_{12/100}) was found to be the most effective for IgG(AF488) infusion. The importance of AF488 labeling of IgGs for providing negative charges on IgGs and the necessity of membrane permeabilizing ability of peptides were also confirmed.

Compared to the liquid droplet (or complex coacervate droplet) formed by FcB(L17E)₃ and IgG(AF488), the PL-L17E_{12/100}/IgG(AF488) complex coacervate had significantly higher stability under the high salt condition – the droplet structures were maintained even in the presence of 2 M NaCl. FRAP experiments also suggested a low fluidity of IgG(AF488) in the coacervate. Therefore, the PL-L17E_{12/100}/IgG(AF488) complex coacervate had more gel-like properties than the FcB(L17E)₃/IgG(AF488) coacervate, presumably because the PL-L17E_{12/100} platform provides more points of electrostatic and hydrophobic interaction with IgG(AF488) to yield denser interaction networks. One of the issues in using liquid droplets and coacervates for drug delivery is the instability of the colloids. The introduction of multivalent membrane permeabilizing peptides into the biocompatible polysaccharide pullulan would provide an appropriate level of entanglement in the complex coacervate with IgG(AF488) to stabilize the colloidal complexes.

The gel-like properties allowed CLSM and SEM imaging to capture structures of the PL-L17E_{12/100}/IgG(AF488) coacervate gel being taken up by the cells, and this was accompanied by dynamic structural changes in the cell membrane. The infusion of IgG(AF488) into the cells

was observed to be synchronized with the cellular uptake steps of the coacervate microgel. The mode of cellular uptake was reminiscent of phagocytosis. However, HeLa is a non-phagocytic cell, revealing the existence of non-canonical or atypical modes of endocytic uptake for the microparticles in non-phagocytic cells.

Although cytosolic IgG(AF488) infusion from the PL-L17E_{12/100}/IgG(AF488) coacervate microgel was less efficient in a serum-containing medium, the use of peptides with stronger membrane permeabilizing abilities, including MLTX and Q21E, was valid even in the presence of serum to bind the intracellular target. Treatment of cells with a complex coacervate gel consisting of PL-MLTX_{12/100} with anti-NPC IgG(AF488) resulted in a marked accumulation of anti-NPC IgG(AF488) on the nuclear membrane. The applicability of the PL-MLTX_{12/100}/IgG(AF488) coacervate microgel for intracellular antibody delivery into primary hepatocytes and iPS cells was also demonstrated. Thus, this study has demonstrated the promise and validity of the delivery concept using the coacervate microgels bearing membrane permeabilizing peptides for basic molecular cell biology studies and for drug delivery. In vivo application of this technology should be considered as a future step.

The formation of complex coacervate microgels and the feasibility of infusing IgGs into cells demonstrated in this study suggest the possibility of establishing systems consisting of different polymers as scaffolds, peptides as membrane permeabilization warheads, and cargo molecules to be delivered into cells with different physicochemical properties and particle sizes, thus establishing novel platforms for cell manipulation and drug delivery. The use of conformationally switchable polymers and peptides that are stimulated by external stimuli may lead to the establishment of stimuli-driven controlled release systems. The cellular machinery allowing cytosolic IgG infusion should also be of interest.

In conclusion, this study has implications not only in providing a novel design platform of stable coacervate for intracellular delivery, but also in elucidating a novel aspect of cellular responses in response to interaction with microgels and eventual cellular uptake. Further studies to improve the applicability and to analyze the uptake mechanisms are ongoing in our laboratory.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental Procedures and Supporting Information Figures S1-S12 including structures, characterization of pullulan conjugates with L17E and related peptides and their complexes with IgGs (PDF), Supporting Information Videos 1-3 showing the IgG(AF488) influx into cells from coacervates formed with PL-L17E_{12/100} (Videos 1 and 2) and PL-MLTX_{12/100} (Video 3) (MP4).

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Author Contributions

J.M. designed and performed the experiments. Y.K. and H.H. provided valuable advice and experimental guidance on liquid-liquid phase separation and intracellular delivery. Y.S. and K.A. provided valuable advice and guidance on the preparation of PL-L17E conjugates and the writing of the paper. A.E. participated in the primary hepatocyte experiments, S.D. and K.T. guided and participated in the iPS cell experiments, and W.X. and T.N. performed the electron microscopy experiments. S.F. supervised the project, designed the experiments, analyzed the data, and wrote the paper with J.M. and Y.K.

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Notes

The authors declare no competing financial interest.

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