

Addition of oligoarginine to a membrane permeabilizing peptide M-lycotoxin facilitates intracellular antibody infusion from microcondensate

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Abstract

Coacervate-based intracellular delivery of biomacromolecules has attracted our attention due to the feasibility of easy condensation of the biomacromolecules and their controllable release. Our laboratory has developed a unique coacervate-based delivery system using the conjugate of the polysaccharide pullulan with membrane-permeabilizing peptides, including L17E and M-lycotoxin, which allows negatively charged immunoglobulin G (IgG) antibodies to enter the cytosol directly through the plasma membranes. Cytosolic IgG distribution is complete within a few minutes after infusion initiation and infusion can be achieved in serum-containing medium. The purpose of this study was to refine this system to reduce the amounts of antibody required while maintaining satisfactory delivery efficiencies. Therefore, pullulan conjugates with M-lycotoxin bearing two to eight arginine residues were designed to enhance the interaction of M-lycotoxin with the cell membrane. The conjugates were able to form microcondensates with Alexa Fluor 488 labeled IgGs. The addition of arginine residues improved the efficiency of cytosolic infusion and successfully reduced the amounts of both antibodies and pullulan-peptide conjugates required for the delivery.

Keywords

peptides, antibodies, coacervate, pullulan, intracellular delivery

Introduction

Coacervation has been used as a means of drug formulation to easily condense and encapsulate molecules of interest.¹⁻² More recently, its properties have also been exploited for the delivery of biomacromolecules, including proteins and nucleic acid polymers, including siRNA and mRNA.³ Successful examples include μm -sized particles composed of a self-assembling histidine-rich peptide that efficiently deliver proteins and polynucleic acids into cells.⁴ Coacervate-based delivery systems with photocontrollable release of the encapsulates have also been reported.⁵ These support the promise of coacervate-based systems for the delivery of biomacromolecules with therapeutic potential into cells. However, coacervates are generally unstable under physiological salt conditions (e.g., $\sim 150\text{ mM NaCl}$), presumably because electrostatic interactions play a role in the formation of the assembly. It is well known that serum often has a negative effect on intracellular delivery by interfering with the interaction of payloads or vehicles.⁶⁻⁷ In fact, there are few reports on coacervate-based delivery that have clearly shown the validity in serum-containing media, which is an important requirement for future in vivo application.

Our research group has developed a unique coacervate-based delivery system to infuse IgG antibodies into cells, which consists of negatively charged IgG and the trimer of the membrane-permeabilizing L17E peptide with cationic charges or the conjugates of L17E with the biocompatible polysaccharide pullulan.⁸⁻⁹ Although other coacervate-based systems have been reported to use endocytosis to deliver the payload into cells, which took a few to several hours for the cytosolic translocation of the cargo proteins, the cytosolic distribution of IgG can be achieved in about one minute with our coacervate-based delivery system. Interaction of the L17E-based coacervate with the target cells resulted in dynamic actin polymerization and alteration of the cell membrane, leading to marked IgG infusion into the cells. Notably, the easy cytosolic IgG delivery is valid in the presence of serum at physiological salt concentration, which is a prerequisite for future in vivo application. Because of these distinctive features of our coacervate-based delivery system, we have named our coacervate-based delivery system "microcondensate" to distinguish it from others. Analysis by fluorescence recovery after photobleaching (FRAP) revealed that the IgGs within the microcondensates exhibited minimal fluidity. This finding suggests that the microcondensates exhibit more gel-like properties than liquid-liquid phase-separated coacervates.⁸⁻⁹

In our previous report on microcondensate-based IgG delivery using conjugates of pullulan with L17E (PL-L17E), we found that the use of pullulan conjugate with M-lycotoxin peptide, which has >20 -fold stronger membrane lytic activity than L17E (PL-MLTX), resulted in the creation of a more efficient microcondensate-based delivery system (Figure S1A).⁹ For microcondensate formation with cationic L17E and MLTX peptide, negative charge addition of IgG by Alexa Fluor 488 (AF488) modification was found to be important. Microcondensate formed with PL-MLTX have achieved delivery of AF488-labeled IgG [IgG(AF488)] with satisfactory efficiency of $\sim 85\%$ of HeLa cells (human cell line derived from cervical cancer) in 1 h by using $\sim 60\text{ }\mu\text{g/mL}$ IgG(AF488) in serum-containing medium. The use of PL-L17E resulted in IgG(AF488) delivery to only $\sim 20\%$ of the cells, suggesting the impact of the PL-MLTX-based microcondensate IgG delivery system. Reducing the amount of antibodies will make this approach more practical for both basic research and potential clinical applications. Therefore, this study aimed to refine this system to reduce the required amounts of antibodies while maintaining satisfactory delivery efficiencies.

Since the use of MLTX instead of L17E significantly improved delivery, a promising approach may be to use peptides that have more membrane interaction compared to MLTX. Arginine-

rich peptides have a high interaction with cell surface molecules.¹⁰⁻¹¹ Therefore, one possible idea to enhance the membrane interaction of MLTX is to add arginine residues. This is reminiscent of our previous report that the addition of four arginine residues to L17E (L17ER4) resulted in more efficient cytosolic protein delivery than using L17E *per se*.¹²⁻¹³ The introduction of positively charged arginine residues should enhance the interaction with negatively charged IgG(AF488). Arginine residues may also promote liquid-liquid phase separation and contribute to coacervate formation.¹⁴⁻¹⁵ In this study, pullulan conjugated with MLTX bearing 2 to 8 arginine residues were prepared and their ability to form microcondensates and to infuse IgG(AF488) into the cytosol was evaluated.

Results and Discussion

Preparation of PL-MLTXRn (n = 2, 4, 6, 8) and Their Microcondensate Forming Ability

Pullulan conjugated with MLTXRn (n = 2, 4, 6, 8) was prepared as in our previous report (Figure S1B).⁹ First, pullulan azide (13.6 azide groups per 100 monosaccharide units) was prepared by introducing azide groups into pullulan (approximately 550 monosaccharide units, ~100 kDa).¹⁶ MLTX peptides bearing 2 to 8 arginine residues together with a Gly-Gly-Cys-amide sequence (MLTXRn-GGC-amide, n = 2, 4, 6, 8) at the C-terminal side were synthesized using the Fmoc solid-phase method (Figure 1A). These peptides were then modified with dibenzocyclooctyne (DBCO)-maleimide and conjugated to pullulan azide via a copper-free click reaction, resulting in PL-MLTXRn (n = 2, 4, 6, 8). Pullulan tagged with MLTX without bearing additional arginine residues was similarly prepared using MLTX bearing a Gly-Gly-Cys-amide at the C-terminus (PL-MLTX, Fig 1A). The peptide-to-pullulan ratio was set at 12 equivalents per 100 monosaccharide units, as this ratio was found to be the most effective for intracellular IgG delivery in our previous report.⁹ The degrees of modification were determined by ¹H NMR, and it were 11.37, 11.89, 11.80, 11.56, and 11.84 for PL-MLTX, PL-MLTXR2, PL-MLTXR4, PL-MLTXR6, and PL-MLTXR8, respectively.

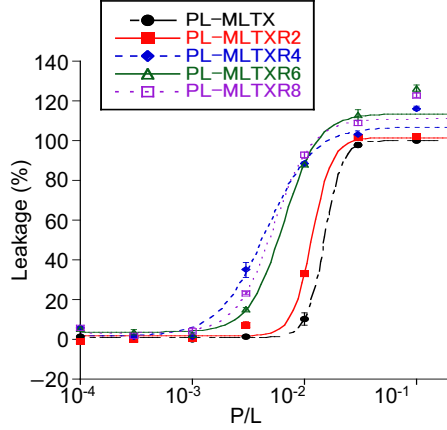
The introduction of arginine residues helps to enhance the membrane disrupting activity of MLTX-modified pullulan. A leakage assay using ANTS/DPX encapsulated liposomes showed that the resulting PL-MLTXRn had a higher membrane permeabilizing activity than PL-MLTX (Figure 1B). ANTS (= 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt) and DPX (= p-xylene-bis-pyridinium bromide) are a fluorescent dye and a quencher, respectively. Perturbation of ANTS/DPX-encapsulated liposomes results in release of ANTS and DPX, which reduces the quenching activity of DPX. The extent of membrane perturbation can be estimated from the increase in ANTS fluorescence.¹⁷ Large unilamellar vesicles (LUVs) of the neutral lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were used as a plasma membrane model.¹⁸ Although no significant difference in leakage activity was observed for PL-MLTXR2 compared to PL-MLTX, PL-MLTXR4 resulted in dye leakage at a significantly lower peptide/lipid ratio than PL-MLTXR2 and PL-MLTX, suggesting that PL-MLTXR4 has a significantly higher membrane permeabilizing ability than these peptides. However, probably because the further increase in the number of arginines was also accompanied by an increase in the hydrophilicity of the peptides, which may have a negative effect on increasing the membrane interaction with POPC, PL-MLTXR6 and PL-MLTXR8 showed almost the same membrane permeability. The P/L ratios at which PL-MLTX, PL-MLTXR2, PL-MLTX4, PL-MLTXR6, and PL-MLTXR8 gave 50% leakage were 1.5×10^{-2} , 1.1×10^{-2} , 4.5×10^{-3} , 6.5×10^{-3} , and 5.4×10^{-3} , respectively.

PL-MLTXRn also formed microcondensates with Alexa Fluor 488-labeled IgGs [IgG(AF488)], and a higher microcondensate forming ability was observed for PL-MLTXRn in the case of n=4, 6, and 8 (Figure 1C). The turbidity of the mixtures and the number of particles in the mixtures, which are measures of microcondensate formation, were then evaluated. Turbidity was measured based on absorbance at 650 nm, while particles were counted by image analysis. The results showed that microcondensates formed with PL-MLTXR4-modified pullulan exhibited higher turbidity and greater number of particles compared to those of PL-MLTXR2 and PL-MLTX. However, no significant increase was observed with the addition of more than four arginine residues.

A

MLTX-GGC-amide : IWLTKFLGKHAAKHLAKQQLSKLGGC-amide
 MLTXR2-GGC-amide: IWLTKFLGKHAAKHLAKQQLSKLRRGGC-amide
 MLTXR4-GGC-amide: IWLTKFLGKHAAKHLAKQQLSKLRRRRGGC-amide
 MLTXR6-GGC-amide: IWLTKFLGKHAAKHLAKQQLSKLRRRRRRGGC-amide
 MLTXR8-GGC-amide: IWLTKFLGKHAAKHLAKQQLSKLRRRRRRRRGGC-amide

B



C

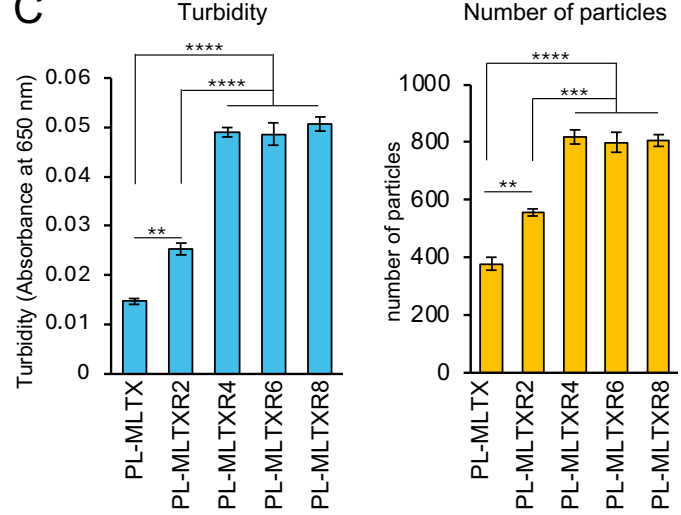


Figure 1. (A) The sequences of MLTX-GGC-amide and MLTXRn-GGC-amide (n = 2, 4, 6, and 8). (B) Liposomal dye leakage by the treatment with conjugates of pullulan with MLTX peptides. P/L indicates the molar concentration ratio of the peptides in the conjugates to POPC. Filled black circle, PL-MLTX; filled red square, PL-MLTXR2; filled blue diamond, PL-MLTXR4; open green triangle, PL-MLTXR6; open purple square, PL-MLTXR8. Lipid concentration; 100 μ M. Temperature; 25°C, pH 7.4. Results are presented as mean $\pm$ standard error (SE) (n=3). (C) Turbidity of the mixture of PL-MLTXRn conjugates (left) and IgG(AF488) and the number of particles (microcondensates) formed (right). Concentration of PL-MLTX and PL-MLTXRn conjugates and IgG(AF488): 2.5 μ M (as peptide concentration) and 50 μ g/mL (molar ratio of MLTXRn:IgG(AF488) = 8:1), respectively. Results are expressed as mean $\pm$ standard error (SE) (n=3). **, p<0.01, ***, p<0.001, ****, p<0.0001 by Tukey-Kramer's honestly significant difference test.

Cytosolic IgG(AF488) Infusion from Microcondensates Formed with PL-MLTXRn (n = 2, 4, 6, 8)

The newly synthesized PL-MLTXRn-modified pullulans had the ability to form microcondensates with IgG(AF488) as shown in Figure 1C, and these microcondensates had the ability to infuse IgG(AF488) into the cytosol. Evaluation of the cytotoxicity of PL-MLTXR2, PL-MLTXR4, PL-MLTXR6, and PL-MLTXR8 showed that none of them exhibited significant cytotoxicity up to 1 μ M (Figure S2). Based on this result, the final concentrations of MLTXRn-modified pullulan and IgG(AF488) were set at 0.5 μ M (from the concentration of MLTXRn) and 10 μ g/mL (molar ratio of MLTXRn:IgG(AF488) = 8:1) for subsequent experiments.

Retention of microcondensate structures in serum-containing media required for future in vivo application was confirmed by confocal laser scanning microscopy (CLSM) (Figure 2A). Here, a mixture of each PL-MLTXRn with the IgG(AF488) was first allowed to form microcondensates by incubation at 23°C for 10 min and then diluted in α -MEM(+) containing 25 mM HEPES (pH 7.4) to obtain the above final concentration for cellular treatment.

Successful cytosolic delivery of IgG(AF488) was observed by CLSM analysis by treating HeLa cells with the microcondensate formed by PL-MLTXR4 and IgG(AF488) for 1 h (Figure 2B). The absence of IgG(AF488) signals in the nucleus suggested the retention of the IgG complex structure even after cytosolic IgG delivery, since the nuclear pore is known to allow only the permeation of proteins with molecular mass $< \sim 40$ kDa, and the mass of IgG (~ 150 kDa) exceeds the threshold. Quantification of cells with cytosolic IgG(AF488) signals indicated that the microcondensate achieved cytosolic IgG(AF488) delivery in nearly 70% of cells (Figure 2C). The lesser extent of IgG(AF488) delivery from microcondensates formed with PL-MLTXR2 and PL-MLTX was observed in ~ 50 and $\sim 30\%$ of cells, respectively. Again, microcondensates formed with PL-MLTXR4, PL-MLTXR6, and PL-MLTXR8 showed comparable levels of delivery. Currently, we do not have the right answer about it. However, one possible explanation is that MLTX is a cationic, amphiphilic lytic peptide, and hydrophobic interactions with membranes play a role in membrane perturbation. The addition of cationic but hydrophilic arginine residues may stimulate the recruitment of MLTX to the membrane due to possible interactions with membrane-associated proteoglycans, as observed for oligoarginines.¹⁰⁻¹¹ Conversely, this increases the hydrophilicity of MLTXRn, reducing the ability to interact with and perturb membranes. These factors may compensate for each other, yielding similar delivery. We currently have no rational answer regarding the factors that enable further improvement of delivery efficiency, and further study is necessary. However, further elaboration on the balance of cationic charges and hydrophobicity, which yield transient yet enhanced membrane permeabilization, may lead to the development of more efficient and effective delivery systems. Considering the ease of preparation of a peptide chain with a minimum number of arginines (n=4) that yielded satisfactory delivery results, PL-MLTXR4 was used for further study.

Increasing the amount of microcondensate added to the cells resulted in increased cytosolic IgG(AF488) infusion. Cytosolic distribution of IgG was observed in $\sim 85\%$ of the cells when the double amount of microcondensate was added to the cells as shown in Figure 2A (i.e., final concentration of MLTXR4 and IgG(AF488): 1 μ M and 20 μ g/mL (molar ratio = 8:1) (Figure 2D, E). Cytosolic IgG infusion was observed in ~ 20 and $\sim 45\%$ of the cells even after treatment with the microcondensate with a final concentration of MLTXR4 of 0.13 and 0.25 μ M,

respectively. To achieve delivery of PL-MLTX (without attachment of arginine residues) to >80% of cells, 3 μ M PL-MLTX and 56 μ g/mL IgG(AF488) were required in our previous study.⁹ These results suggest that under serum-containing conditions, PL-MLTXR4 reduces the amount of antibody required to achieve a similar delivery efficiency as PL-MLTX to approximately 1/3.

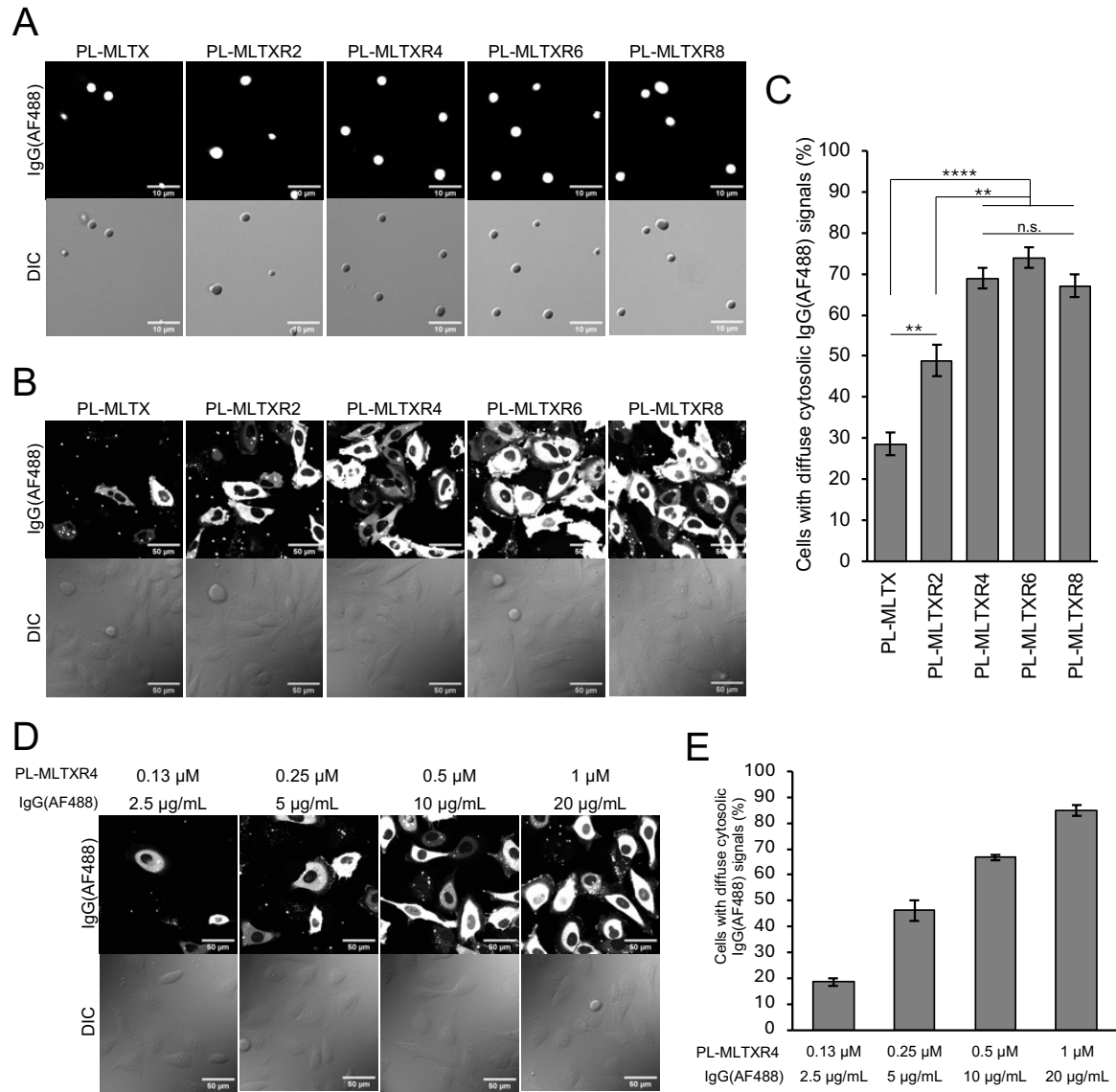


Figure 2. (A) Microcondensate formation of PL-MLTX and PL-MLTXRn with IgG(AF488). Scale bars, 10 μ m. (B) CLSM observation of HeLa cells treated with PL-MLTXRn/IgG(AF488) microcondensates for 1 h. Scale bars, 50 μ m. (C) Percentage of cells with diffuse cytosolic IgG(AF488) signals. Results are expressed as mean $\pm$ SE (n=3). **, p<0.01, ****, p<0.0001, n.s.; not significantly different by Tukey-Kramer's honestly significant difference test. Final concentrations of PL-MLTX/PL-MLTXRn and IgG(AF488) used for (A), (B) and (C): 0.5 μ M (as peptide concentration) and 10 μ g/mL, respectively. (D) Cytosolic IgG(AF488) distribution in HeLa cells treated with PL-MLTXR4/IgG(AF488) at different concentrations. Scale bars, 50 μ m. (E) The percentage of cells with diffuse cytosolic IgG(AF488) signals. Results are expressed as mean $\pm$ SE (n=3).

Modes of Cytosolic IgG(AF488) Infusion from PL-MLTXR4-based Microcondensate

Our previous studies using FcB(L17E)₃ and PL-L17E/MLTX-based microcondensates showed that these microcondensates share the feature of allowing immediate cytosolic distribution of IgG(AF488) within one or a few minutes after the microcondensates are attached to cells.⁸⁻⁹ This feature was also observed for this PL-MLTXR4-based microcondensate. Time-lapse imaging showed that the cytosolic infusion of IgG(AF488) started after the attachment of microcondensates to cells (arrowhead, Figure 3A), and the cytosolic distribution was achieved in one minute.

Cytosolic infusion of IgG(AF488) using PL-MLTXR4-based microcondensate was achieved in cells other than HeLa cells, as in the case of other microcondensates⁸⁻⁹ (Figure 3B). Using microcondensates formed from 0.5 μ M PL-MLTXR4 and 10 μ g/mL IgG(AF488) resulted in cytosolic IgG(AF488) distribution of 60, 50, and 25% in A549 (human cell line derived from lung cancer), HT1080 (human sarcoma cell line with activated N-Ras oncogene), and MIA PaCa-2 (human cell line derived from pancreatic cancer), respectively (Figure 3C). The percentages obtained with PL-MLTXR4-based microcondensate were 3- to 4-fold higher than those obtained with PL-MLTX-based microcondensate, demonstrating the superiority of PL-MLTXR4-based microcondensate as an intracellular IgG delivery system.

Due to its similarity to the modes of cytosolic infusion of IgG(AF488) using a microcondensate formed with PL-L17E and PL-MLTX,⁸⁻⁹ the PL-MLTXR4-based microcondensate containing IgG(AF488) can be taken up by cells trapped by the plasma membrane. Subsequent membrane perturbation by MLTXR4 should lead to the infusion of IgG(AF488) into the cytosol. The bright, sphere-like signals indicated by the arrowheads in Figure 3A at 32 and 33 min may represent a PL-MLTXR4-based microcondensate structure brought into cells while trapped in large endosome-like membrane structures, as reported in our previous study.⁹ Because the microcondensate exhibited gel-like properties and contained a high concentration of IgG(AF488), the bright signal could persist even after a significant portion of the IgG(AF488) was released into the cytosol.

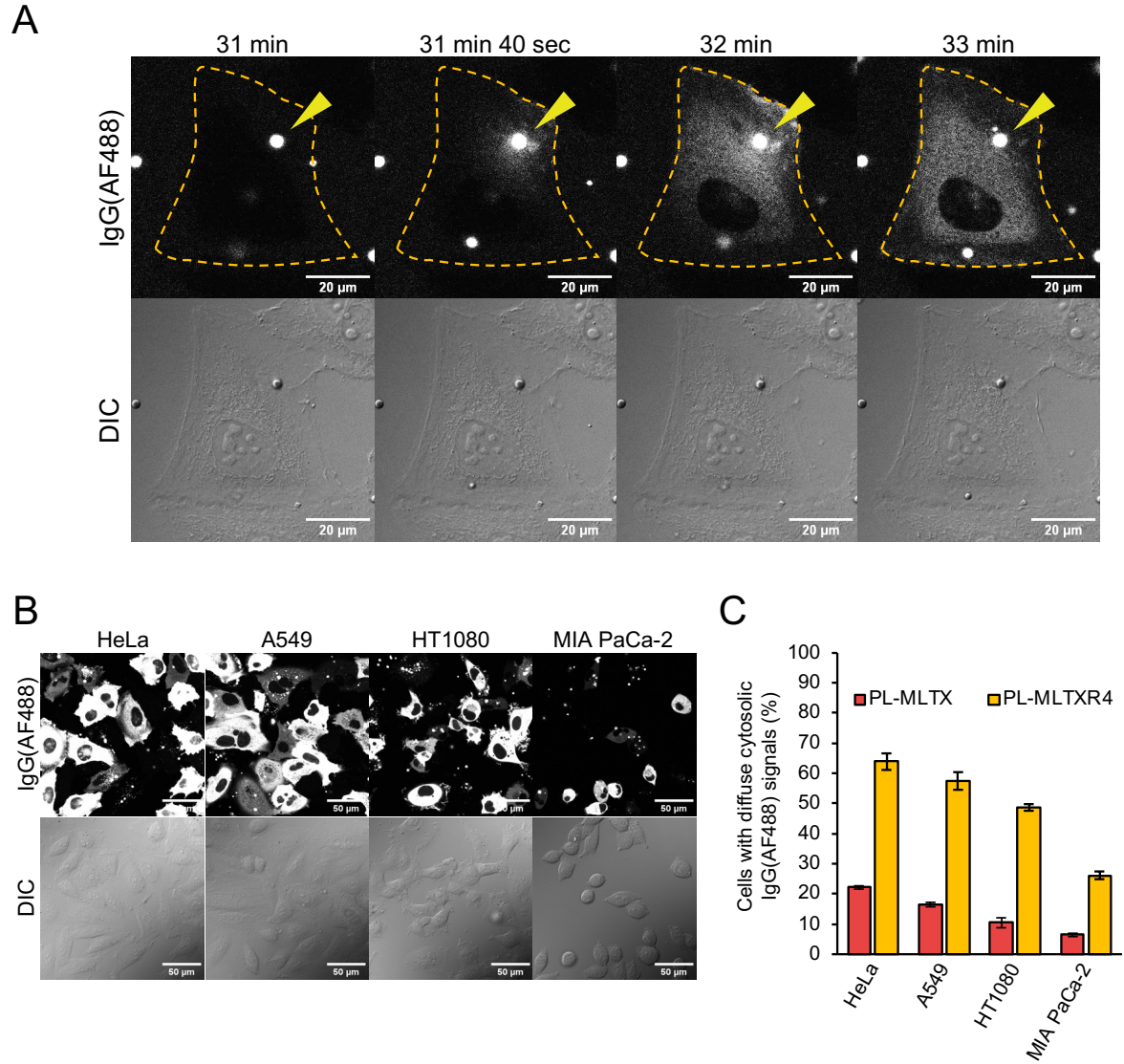


Figure 3. (A) Time-lapse imaging of cells treated with microcondensate from of PL-MLTXR4 and IgG(AF488). Arrowheads indicate a particle-like structure that interacts with the periphery of a cell, resulting in the distribution of IgG(AF488) signals within the cells (the cell boundary is highlighted with a dotted line). PL-MLTXR4 and IgG(AF488) concentrations; 0.5 μ M (as MLTXR4 concentration) and 10 μ g/mL, respectively. Scale bars; 20 μ m. (B) Cytosolic IgG(AF488) distribution in different cell lines treated with microcondensate formed from PL-MLTXR4 with IgG(AF488). Concentrations of PL-MLTXR4 and IgG(AF488) used for complex formation: 0.5 μ M (as MLTXR4) and 10 μ g/mL, respectively. Scale bars, 50 μ m. (C) The percentage of cells with diffuse cytosolic IgG(AF488) signals. Concentrations of PL-MLTX or PL-MLTXR4 and IgG(AF488) used for complex formation: 0.5 μ M (as MLTXR4) and 10 μ g/mL, respectively. Results are expressed as mean $\pm$ SE (n=3).

Retention of Target Recognition Ability of Delivered IgG

IgG thus delivered into cells by PL-MLTXR4-based microcondensate retained target recognition ability. Alexa Fluor 488-labeled mouse anti-nuclear pore complex (NPC) IgG [anti-NPC IgG(AF488)] was mixed with PL-MLTXR4 to form microcondensate, followed by the addition of α -MEM(+) containing 25 mM HEPES (pH 7.4) prior to treatment with HeLa cells for 1 h. A marked accumulation of AF488 signals was obtained around the nucleus or nuclear membranes, suggesting that IgG was delivered to the cytosol using PL-MLTXR4-based microcondensate while retaining its recognition ability (Figure 4).

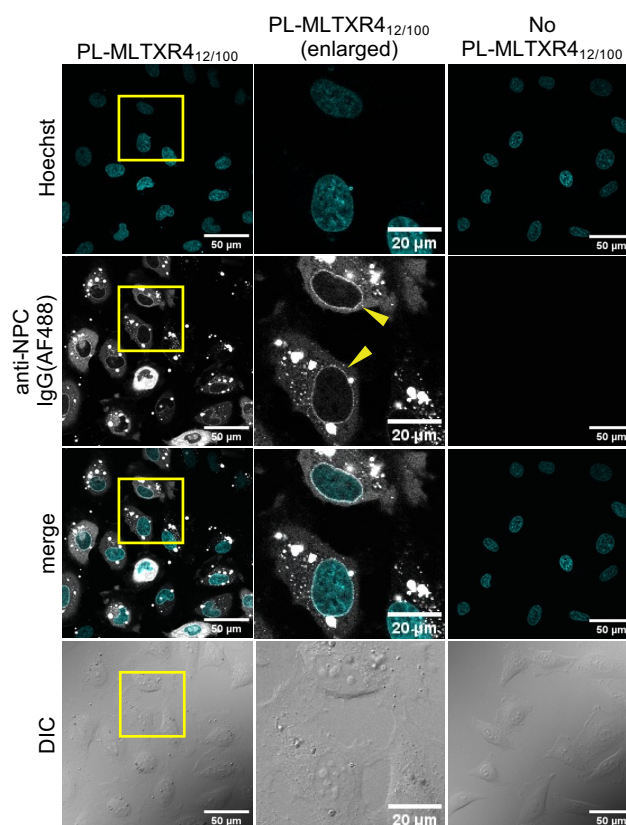


Figure 4. Accumulation of anti-nuclear pore complex (NPC) IgG(AF488) infused from the microcondensate formed from PL-MLTXR4 with anti-NPC IgG(AF488) after incubation in serum-containing α -MEM for 1 h. Concentrations of PL-MLTXR4 and anti-NPC IgG(AF488) used for complex formation: 0.5 μ M (as peptide concentration) and 10 μ g/mL, respectively.

Conclusion

The aim of this study was to refine the microcondensate-based delivery system using PL-MLTX and to reduce the amount of antibody required while maintaining satisfactory delivery efficiencies. Therefore, MLTX peptides with 2, 4, 6, and 8 residues of arginine at the C-terminus were prepared and conjugated with pullulan. PL-MLTXR4 was selected as the most suitable. Comparable efficacy of IgG(AF488) delivery was achieved using almost 1/3 of the amount of PL-MLTXR4 and IgG(AF488) compared to the use of PL-MLTX in serum containing media. The targeting of IgG(AF488) to the intracellular compartment was confirmed by the use of anti-NPC IgG(AF488).

Method

Peptide synthesis

The peptide chain of each peptide was constructed by Fmoc solid-phase peptide synthesis using a 1-[bis(dimethylamino)methylene]-*1H*-benzotriazolium-3-oxide hexafluorophosphate (HBTU)/1-hydroxybenzotriazol (HOBt)/*N,N*-diisopropylethylamine (DIEA) coupling system on Rink amide resin (TGS-RAM, Shimadzu) using a Biotage Syro I peptide synthesizer. Fmoc amino acid derivatives were purchased from Peptide Institute or Watanabe Chemical Industries, respectively. After the peptide chains were constructed, the protected peptide resin was treated with a mixture of trifluoroacetic acid (TFA) and 1,2-ethanedithiol (EDT) (95:5) for 3 h at room temperature (~23°C) to cleave from the resin and deprotect the side chain protecting groups. The deprotected peptide was then purified by reversed-phase high performance liquid chromatography (RP-HPLC). The mass of the obtained peptide was confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS): MLTX-GGC-amide (IWL TALKFLGKHA AKHLAKQQLSKL-GGC-amide) 3059.99 [calcd for (M+H)⁺ 3059.78]; MLTXR2-GGC-amide (IWL TALKFLGKHA AKHLAKQQLSKLRR-GGC-amide) 3372.28 [calcd for (M+H)⁺ 3371.98]; MLTXR4-GGC-amide (IWL TALKFLGKHA AKHLAKQQLSKLRRRR-GGC-amide) 3683.29 [calcd for (M+H)⁺ 3684.19]; MLTXR6-GGC-amide (IWL TALKFLGKHA AKHLAKQQLSKLRRRRRR-GGC-amide) 3996.58 [calcd for (M+H)⁺ 3996.39]; MLTXR8-GGC-amide (IWL TALKFLGKHA AKHLAKQQLSKLRRRRRRRR-GGC-amide) 4308.90 [calcd for (M+H)⁺ 4308.59].

Preparation of conjugates of pullulan with L17E (PL-L17Es) and other peptides

(i) Preparation of DBCO-modified peptides

MLTX-GGC-amide and MLTXR_n-GGC-amide (n = 2, 4, 6, and 8) were treated with DBCO-maleimide (Tokyo Chemical Industry, 1.2 eq.) in dimethylformamide (DMF)-methanol (1:1) in the presence of *N*-methylmorpholine (NMM) for 30 min. Each DBCO-modified peptide was then purified by HPLC to yield MLTX(DBCO) and MLTXR_n(DBCO) (n = 2, 4, 6, and 8), respectively. The concentration of the peptide solution was determined by absorbance at 280 nm using the molar extinction coefficient (ϵ) = 16,500 cm⁻¹ M⁻¹, which is the sum of the ϵ for tryptophan and DBCO (ϵ = 5,500 and 11,000 cm⁻¹ M⁻¹, respectively).¹⁹⁻²⁰ The masses of the DBCO-modified peptides obtained were confirmed by MALDI-TOF MS: MLTX(DBCO) 3487.42 [calcd. for (M+H)⁺ 3486.94]; MLTXR2(DBCO) 3799.31 [calcd. For (M+H)⁺ 3799.14]; MLTXR4(DBCO) 4111.94 [calcd. for (M+H)⁺ 4111.34]; MLTXR6(DBCO) 4422.91 [calcd. for (M+H)⁺ 4423.54]; MLTXR8(DBCO) 4735.37 [calcd. for (M+H)⁺ 4735.74].

(ii) Preparation of pullulan-azide

Pullulan-azide was prepared as reported.¹⁶ Briefly, pullulan was dissolved in super dehydrated dimethyl sulfoxide (super dehydrated DMSO, FUJIFILM Wako Chemicals) to prepare a solution of about 20 mg/mL, followed by the addition of 1,1'-carbonyldiimidazole (CDI, Tokyo Chemical Industry) (20 equivalents to 100 glucose units) in super dehydrated DMSO. The mixture was stirred at room temperature (~23°C) for 16 h. After the addition of 3-azidopropylamine (Tokyo Chemical Industry) (40 eq), the mixture was stirred for 24 h, then dialyzed three times against pure water and lyophilized. Pullulan-azide (containing 13.6 azide

units/100 glucose units) was thus obtained. The modification ratio of the azide units to 100 glucose units was determined by ^1H NMR measurement as reported.⁹ Pullulan-azide was dissolved with DMSO- d_6 (Eurisotop) (600 μL) and D_2O (Tokyo Chemical Industry) (30 μL). The solution was transferred to the NMR tube (NES-600, OPTIMA) and measured with ECA600 (JEOL) (600 MHz, 90°C, 15 s relaxation delay).

(iii) Preparation of PL-peptides and conjugates of pullulan with other peptides

Each DBCO-modified peptide was added to the pullulan-azide obtained above (12 eq of DBCO-modified peptides to 100 glucose units of pullulan-azide) in H_2O /DMSO and stirred overnight at room temperature ($\sim 23^\circ\text{C}$). Unreacted peptides and DMSO were removed, and the solvent was exchanged with MilliQ water by ultrafiltration using Amicon Ultra-0.5 (molecular weight cutoff: 30,000, Merck), and then the solution of each PL-peptides (PL-MLTX, PL-MLTXR2, PL-MLTXR4, PL-MLTXR6, PL-MLTXR8) was lyophilized. The modification ratios of the peptides to pullulan were determined by measuring the ^1H NMR as reported.⁹ Briefly, the integration ratio of the α -anomeric protons of pullulan and that of the protons at the 6-position of the tryptophan side chain were compared. Signals derived from DBCO moieties overlapping with α -anomeric protons were subtracted. The concentration of the PL-peptides solution was determined by absorbance at 280 nm using the molar extinction coefficient (ϵ) = $7300\text{ cm}^{-1}\text{ M}^{-1}$.⁹

IgG(AF488)

For Alexa Fluor 488 modification of human serum IgG, IgG (FUJIFILM Wako Chemicals, 143-09501; 10 mg/mL, dissolved in 10 mM sodium phosphate (pH 7.2) containing 150 mM NaCl, 0.05 w/v% sodium azide) (100 μL), phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(-)] (340 μL), 1 M NaHCO_3 (aqueous solution) (50 μL), and Alexa Fluor 488 5-SDP ester (Thermo Fisher Scientific) in DMSO (10 mg/mL, 10 μL) were mixed and allowed to react for 1 h at room temperature ($\sim 23^\circ\text{C}$) with gentle mixing. IgG(AF488) was purified by gel filtration on a PD-10 column (Cytiva) followed by ultrafiltration using Amicon Ultra-4 (mwco: 50,000). The concentration of IgG was determined by the absorbance at 280 nm using $\epsilon = 2.1 \times 10^5\text{ cm}^{-1}\text{ M}^{-1}$ and the extent of AF488 modification was determined by the concentration of Alexa Fluor 488 from the absorbance at 495 nm using $\epsilon = 7.1 \times 10^4\text{ cm}^{-1}\text{ M}^{-1}$, divided by the IgG concentration.²¹⁻²² IgG modified with 7-9 Alexa Fluor 488 units was used for the study.

For modification of mouse anti-nuclear pore complex IgG, IgG solution (1 mg/mL, BioLegend, Cat. No. 902902) (100 μL), PBS(-) (32 μL), 1 M NaHCO_3 (aqueous solution, 15 μL), and Alexa Fluor 488 5-SDP ester (Thermo Fisher Scientific) in DMSO (10 mg/mL, 3 μL) were mixed, and the procedures described above were followed.

Liposome Preparation

Large unilamellar vesicles (LUVs) composed of 100% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (NOF) were prepared. A chloroform solution of 10 mg POPC (800 μL) was added to a round-bottomed flask, and a lipid thin film was formed on the surface of the flask using a rotary evaporator. The lipid thin film was then vacuum dried and hydrated with 10 mM MES-buffered saline (MBS) (10 mM MES containing 150 mM NaCl, pH 7.4) containing the fluorescent dye 8-aminonaphthalene-1,2,3-trisulfonic acid (ANTS, 12.5

mM) and its quencher *p*-xylene-bis(pyridinium) bromide (DPX, 45.0 mM). After five freeze-thaw cycles, the lipid suspension was extruded through two Nucleopore Track-Etch Membranes (0.1 μ m, Cytiva) using a LiposoFast extruder system (Avestin) with 11 passes to achieve a uniform particle size of 100 nm. The resulting LUVs encapsulating ANTS/DPX were purified from free dye via size exclusion chromatography using a PD-10 desalting column (GE Healthcare). The final concentration of LUVs was determined based on the phospholipid content using the LabAssay Phospholipid Kit (FUJIFILM Wako Pure Chemical).

ANTS/DPX Leakage Assay

The lipid membrane destabilization activity of PL-peptides was evaluated by measuring the fluorescence intensity of ANTS released from LUVs. PL-peptides were incubated with 100 μ M (as lipid concentration) LUVs in 10 mM MBS (pH 7.4) at 37°C for 1 hour. Fluorescence was measured using a Nivo microplate reader (PerkinElmer) with an excitation wavelength of 355 nm and an emission wavelength of 530 nm. The membrane destabilizing activity of PL-peptides was evaluated based on the leakage percentage (%) using the following formula, where $F_{\max}$ represents the fluorescence intensity upon the addition of the surfactant Triton X-100 [final concentration 0.1% (v/v)] and F_{blank} represents the fluorescence intensity in the absence of PL-peptides. The concentration of the PL-peptides was based on that of each peptide conjugated to pullulan.

$$\text{leakage (\%)} = \frac{F_{\text{obs}} - F_{\text{blank}}}{F_{\text{max}} - F_{\text{blank}}} \times 100$$

Evaluation of Microcondensate Forming Ability

The microcondensate formation ability of each PL-peptide with IgG(AF488) was evaluated through turbidity measurements and particle count by fluorescence microscopy observations. A mixture of PL-MLTX or PL-MLTXR_n (n = 2, 4, 6, and 8) in MBS (pH 5.0) (75 μ M as peptide concentration, 1.7 μ L) and IgG(AF488) in MBS (pH 5.0) (1.5 mg/mL, 1.7 μ L) was incubated at room temperature (~23°C) for 10 mins. Then, 47 μ L of α -MEM(+) containing 25 mM HEPES (pH 7.4) was added to each sample, which was transferred to a 96-well half-area microplate (Corning, 3695), and absorbance at 650 nm was measured as an indicator of turbidity. After measurement, the mixtures were left for 30 min to allow sedimentation of the coacervates, followed by imaging and particle counting using all-in-one fluorescence microscope (BZ-X810, KEYENCE) equipped with an image cytometer module (BZ-H4XI, KEYENCE) and a hybrid cell count application (BZ-H4A, KEYENCE).

Cell Culture

HeLa cells (human epithelial carcinoma cell line) were obtained from the European Collection of Authenticated Cell Cultures (ECACC). HT1080 cells (human fibrosarcoma cell line) were obtained from the JCRB Cell Bank. A549 cells (human lung cancer cell line) and MIA PaCa-2 cells (human pancreatic adenocarcinoma cell line) were obtained from the RIKEN BioResource Research Center (RIKEN BRC). Irrespective of the cell line, cells were cultured at 37°C under 5% CO₂ and were passaged every 2–3 days. HeLa cells were cultured in α -minimum essential medium (α -MEM, FUJIFILM Wako Chemicals) containing 10% (v/v) heat-

inactivated bovine serum (BS, Gibco). HT1080, A549, and MIA PaCa-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose, FUJIFILM Wako Chemicals) with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco).

Cytotoxicity Assay

The cytotoxicity of each PL-peptide was evaluated using the Cell Counting Kit-8 (Dojindo). HeLa cells were seeded to a 96-well plate (1.0×10^4 cells/well) and incubated overnight under the above culture conditions. Each PL-peptide (750 μ M as peptide concentration, 11 μ L) was diluted with α -MEM(+) containing 25 mM HEPES (pH 7.4) to a final volume of 270 μ L, resulting in a final concentration of 10 μ M. Further dilution with α -MEM(+) containing 25 mM HEPES (pH 7.4) was performed to prepare 160 μ L of PL-peptide solutions at 5, 3, 1, 0.5, and 0.3 μ M (as peptide concentration). After the cells were washed with PBS(–), each sample (50 μ L/well) was added to the cells, and incubated for 1 hour under the culture conditions. The cells were then washed with PBS(–) before adding a mixture of α -MEM(+) containing 25 mM HEPES (pH 7.4) (91 μ L) and WST-8 solution (9 μ L). After incubation at 37°C for 1 h, the absorbance of formazan at 450 nm (with background subtraction at 600 nm) was measured.

Cytosolic Delivery Assay of IgG

HeLa cells were seeded (2.0×10^5 cells/dish) in a 35 mm glass-bottomed dish and incubated overnight at 37°C under 5% CO₂ to 80–90% confluence. A mixture of 1 μ L of PL-peptides in MBS (75 μ M as peptide concentration, pH 5.0) and 1 μ L of IgG(AF488) in MBS (1.5 mg/mL, pH 5.0) was incubated at room temperature (~23°C) for 10 min. Then, α -MEM(+) containing 25 mM HEPES (pH 7.4) was added to the mixture (final volume: 150 μ L) prior to cell treatment. After the cells were washed twice with PBS(–), the prepared mixture was added, and the cells were incubated for 1 h (37°C, 5% CO₂). The sample solution was then removed, and the cells were washed twice with PBS(–) containing 0.5 mg/mL heparin, followed by the addition of α -MEM(+) containing 25 mM HEPES (pH 7.4). Cells were observed with a confocal microscope (FV3000, Olympus).

To evaluate the concentration-dependent change in efficiency, mixtures of PL-MTXR4 in MBS (75 μ M as peptide concentration, pH 5.0) and IgG(AF488) in MBS (1.5 mg/mL, pH 5.0) were similarly prepared and incubated for 10 min, and then diluted up to 150 μ L with varying volumes of α -MEM(+) containing 25 mM HEPES (pH 7.4) to yield the final concentrations of PL-MLTXR4 of 1 μ M, 0.5 μ M, 0.25 μ M, and 0.13 μ M, and of IgG(AF488) of 20 μ g/mL, 10 μ g/mL, 5 μ g/mL, and 2.5 μ g/mL, respectively.

To evaluate the target recognition ability of the delivered IgG, anti-NPC IgG(AF488) was used, and cells were similarly treated with the mixture of PL-MLTXR4 and anti-NPC IgG(AF488). After two washes with PBS(–) containing 0.5 mg/mL heparin, Hoechst 33342 (5 μ g/mL) in α -MEM(+) containing 25 mM HEPES (pH 7.4) was added to the cells and incubated for 5 min for nuclear staining. Cells were washed and observed with an Olympus FV3000 confocal microscope after the addition of α -MEM(+) containing 25 mM HEPES (pH 7.4).

Delivery assays in cell lines other than HeLa were performed similarly using DMEM(+) containing 25 mM HEPES (pH 7.4).

Quantification of IgG Delivery Efficiency

HeLa cells were seeded (8.0×10^4 cells/well) in a 24-well plate (Thermo Fisher Scientific) and incubated overnight at 37°C under 5% CO₂ until 80–90% confluence was reached. A mixture of 5.2 µL of PL-peptides in MBS (75 µM as peptide concentration, pH 5.0) and 5.2 µL of IgG(AF488) in MBS (1.5 mg/mL, pH 5.0) was incubated at room temperature (~23°C) for 10 min. Then, α-MEM(+) containing 25 mM HEPES (pH 7.4) was added to the mixture (final volume: 780 µL). After the cells were washed twice with PBS(–), the prepared mixture was added (250 µL/well), and the cells were incubated for 1 h (37°C, 5% CO₂). The sample solution was then removed, and the cells were washed twice with PBS(–) containing 0.5 mg/mL heparin. Hoechst 33342 (5 µg/mL in PBS(–)) was added and the cells were incubated for 5 min. The cells were washed and fixed by 4% paraformaldehyde (PFA) in PBS(–) and incubated for 10 min. After PFA was removed and PBS(–) was added, images were captured using a BZ-X810 fluorescence microscope equipped with an image cytometer module. The acquired images were analyzed to count the number of cells bearing cytosolic diffuse AF488 signals using a hybrid cell count application, which means that IgG(AF488) was successfully delivered into the cytosol, and the percentage to all cells was calculated using the image cytometry analysis software of the BZ-X810.

To evaluate the concentration dependence of cytosolic IgG(AF488) delivery efficiency, 780 µL of each sample containing different concentrations of PL-MLTXR4 and IgG(AF488) were prepared as described above.

Delivery assays in cell lines other than HeLa were performed similarly using DMEM(+) containing 25 mM HEPES (pH 7.4).

Time-Lapse Imaging

HeLa cells were seeded (2.0×10^5 cells/dish) into a 35 mm glass-bottom dish and cultured overnight at 37°C in a 5% CO₂ atmosphere until they reached 80–90% confluence. After the cells were washed twice with PBS(–), α-MEM(+) containing 25 mM HEPES (pH 7.4) was added, and the dish was placed in an STX stage-top incubator (TOKAI HIT), set at 37°C with 5% CO₂, and mounted on a FV3000 confocal microscope (Olympus). Time-lapse imaging was performed at 20-second intervals. After the initial imaging period (3 min), the culture medium was replaced with microcondensates formed from IgG(AF488) (10 µg/mL) and PL-MLTXR4 (0.5 µM as peptide concentration) in α-MEM(+) containing 25 mM HEPES (pH 7.4) and imaging was continued.

ASSOCIATED CONTENT

Supporting Information

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

Supporting Information Figures S1 and S2 including schematic representation of cytosolic IgG infusion from microcondensates, preparation of pullulan-MLTXRn conjugates (PL-MLTXRn, $n = 2, 4, 6$, and 8), and cytotoxicity of PL-MLTXRn ($n=2, 4, 6$, and 8).

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Author Contributions

J.M. designed and performed the experiments. Y.K. provided valuable advice and experimental guidance on microcondensate formation and intracellular delivery. Y.F. is involved in preparation of MLTXRn ($n=2, 4, 6$, and 8) and their conjugates with pullulan. Y.S. and K.A. provided valuable advice and guidance on the preparation of PL-MLTX conjugates

and the writing of the paper. 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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