Self-Degradable Lipid-Like Materials Based on “Hydrolysis accelerated by the intra-Particle Enrichment of Reactant (HyPER)” for Messenger RNA Delivery

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RNA-based therapeutics is a promising approach for curing intractable diseases by manipulating various cellular functions. For eliciting RNA (i.e., mRNA and siRNA) functions successfully, the RNA in the extracellular space must be protected and it must be delivered to the cytoplasm. In this study, the development of a self-degradable lipid-like material that functions to accelerate the collapse of lipid nanoparticles (LNPs) and the release of RNA into cytoplasm is reported. The self-degradability is based on a unique reaction “Hydrolysis accelerated by intra-Particle Enrichment of Reactant (HyPER).” In this reaction, a disulfide bond and a phenyl ester are essential structural components: concentrated hydrophobic thiols that are produced by the cleavage of the disulfide bonds in the LNPs drive an intraparticle nucleophilic attack to the phenyl ester linker, which results in further degradation. An oleic acid-scaffold lipid-like material that mounts all of these units (ssPalmO-Phe) shows superior transfection efficiency to nondegradable or conventional materials. The insertion of the aromatic ring is unexpectedly revealed to contribute to the enhancement of endosomal escape. Since the intracellular trafficking is a sequential process that includes cellular uptake, endosomal escape, the release of mRNA, and translation, the improvement in each process synergistically enhances the gene expression.

1. Introduction

Messenger RNA (mRNA) is an ideal biological tool for the replacement/complementation of desired proteins to cells, since it is free from risks of genomic integration and insertional mutagenesis.[1–3] The intrinsically transient nature of the expression of the mRNA is also beneficial for avoiding the persistent expression of therapeutic proteins such as antigens,[4] antiapoptotic proteins,[5] transcription factors,[6,7] and nucleases for genome editing.[8] However, the cytoplasmic delivery of the mRNA is significantly hampered by the presence of extracellular ribonucleases and plasma/endosomal membranes, since the mRNA is enzymatically vulnerable and highly hydrophilic molecule. The development of an efficient mRNA delivery system is thus prerequisite for its therapeutic application.
Lipid nanoparticles (LNPs) composed of ionizable lipids, artificial hydrophobic materials with tertiary amine structures, represent a breakthrough technology for RNA delivery. Such LNPs make up the world’s first clinical application of a small-interfering RNA (siRNA).\[^{[8-12]}\] In this system, siRNA molecules are encapsulated in the interior of the LNPs to protect it from enzymatic degradation and for controlling its pharmacokinetics. The LNPs are designed so as to be neutral to avoid undesired electrostatic interactions with biomaterials in biological fluids such as erythrocytes and/or platelets.\[^{[13]}\] Once the LNPs are taken up by cells via endocytosis, the tertiary amines become protonated in response to the acidic pH during the endosomal maturation to lysosomes. The protonation results in the development of a cationic charge on the surface of the LNPs. The cationic charge then enhances interactions between the LNPs and anionic endosomal membranes, a process that is required for a successful endosomal escape of the siRNA to the cytoplasm.\[^{[14,15]}\] Since the target organelle of the mRNA is also the cytoplasm, as in the case of siRNA, the concept of acidic pH dependent endosomal escape has been widely applied to various types of ionizable lipids or lipid-like materials for mRNA delivery systems.\[^{[2,16-24]}\]

The expression of the mRNA is profoundly affected by the efficiency of translation. This notion is supported by the observation that the preassembly of an mRNA with recombinant eIF4E, a component of a translation initiation complex that recognizes 5′-cap, significantly enhances transgene activity. The half-life of the mRNA formulated in a polyplex-type carrier is greatly dependent on the intraparticle Enrichment of Reactant (HyPER). In this reaction, a thiol group (a nucleophile) and a phenyl ester (an electrophile) are both concentrated in the limited intraparticle space, which serves to accelerate a nucleophilic substitution reaction. This irreversible chemical reaction triggers the self-degradation, collapse of the nanoparticle, and the subsequent release of the mRNA. The in vitro and in vivo transgene activity of the ssPalmO-Phe was compared to that of nondegradable counterparts, commercially available reagents, and conventional ionizable lipids. We also report that the insertion of an aromatic ring resulted in a drastic improvement in the acidic pH-triggered endosomal membrane destabilization activity.

2. Results and Discussion

2.1. Design and Degradability of the ssPalmO-Phe

2.1.1. Molecular Design of the ssPalmO-Phe

A schematic diagram illustrating the HyPER reaction is shown in Figure 1b. In the initiation process, hydrophobic thiols are generated by the reductive cleavage of the disulfide bonds of the ssPalms. The continuous reducing reaction increases the intraparticle concentration of the thiols. The enriched thiols eventually attack labile bonds in the LNPs, resulting in their breakdown. We unexpectedly found this reaction when palmitic acid-4-methylumbelliferone ester was loaded to the LNPssPalm (C16-4MU, Figure 1c). The encapsulated 4MU was released from the LNPs as phenolic molecules when they were exposed to a 10 × 10^{-3} μM glutathione solution (Figure S1, Supporting Information). Moreover, this GSH-dependent hydrolysis of the 4MU ester was observed only when they were loaded in the LNPssPalm (Figure S1, Supporting Information): in LNPs without ssPalm (i.e., LNP_{DODAP} (Figures S1 and S2a, Supporting Information) and liposome composed of EPC/cholesterol (Figure S1, Supporting Information)), no hydrolysis was detected in the presence of GSH. These data indicate that the GSH cannot directly cleave the phenyl ester that is located in the particle. This can partly be explained by the cLogP value, a calculated index of logarithm of octanol–water partition coefficient, of GSH (≈6). A strongly negative cLogP value means that the GSH is too hydrophilic to be distributed to the hydrophobic...
region of the nanoparticles. Thus, the GSH-derived thiols are unable to gain access to the phenyl esters in intraparticle region. Such a phenyl ester-dependent cleavage in LNPs was also observed at the molecular level (Figure S2a,b, Supporting Information). The rate of release of 4MU was increased along with the increase in ssPalm content in the LNPs (Figure S2c,d, Supporting Information). Furthermore, a similar degradation reaction was observed in the case of cholesteryl-hemisuccinate esters of mono-chloro-substituted or nonsubstituted phenols (Figure S3, Supporting Information). We thus concluded that the phenyl ester moiety is a suitable substrate for the HyPER reaction.

The structures of the ssPalms used in the present study are shown in Figure 1a. A conventional ssPalm with oleic acid scaffolds (ssPalmO) was selected as a seed material for molecular tuning, because of its less inflammatory nature.[43] As a self-degradable material, a phenyl ester unit was inserted as a linker between the amine and the oleic acid to develop the ssPalmO-Phe. A benzyl ester was inserted as a linker (ssPalmO-Ben) as a nondegradable counterpart.

2.1.2. Self-Degradation of the ssPalmO-Phe via the HyPER Reaction

LNPs composed of the ssPalmO-Ben or the ssPalmO-Phe (LNPssPalmO-Ben or LNPssPalmO-Phe, respectively) were treated with GSH (Figure 2). To avoid aggregation, the LNPs were stabilized by lipid-conjugated polyethylene glycol (DMG-PEG2000, 3 mol% of total lipids). At the indicated time points, the degradation of the ssPalms was monitored by high-performance liquid chromatography (HPLC). Representative HPLC charts are shown in Figure S4 (Supporting Information). In the absence of the reducing agent, the ssPalmO-Ben and the ssPalmO-Phe were stable throughout the experiments (filled circles). In the case of the ssPalmO-Ben, incubation with the GSH resulted in the partial cleavage of the dimer to the monomer and its GSH adduct (Figure 2a). The monomer/dimer ratio reached a plateau within 5 min after the beginning of the reaction. This behavior indicates that the cleavage of the ssPalmO-Ben (reduction) and its reverse reaction (oxidation) reaches equilibrium. At the steady state, ≈75% of the ssPalmO-Ben continues to remain in the oxidized dimeric form. On the other hand, the amount of the ssPalmO-Phe decreased steadily and monotonically under the same reducing conditions (Figure 2b). After 12 h of incubation, the parent ssPalmO-Phe, the reduced form of ssPalmO-Phe (monomer), and its GSH adduct had completely degraded. For a further confirmation, the LNPs were treated with another reducing agent, dithiothreitol (DTT). Treatment of ssPalmO-Ben with the DTT resulted in nearly complete formation of the reduced form at 1 h after the treatment (Figure S5a, Supporting Information). However, the amount of the ssPalmO-Ben (dimer) were recovered in a time-dependent manner, probably due to the reoxidation reaction. In contrast, the ssPalmO-Phe and the cleaved compounds were completely degraded within 6 h (Figure S5b, Supporting Information). The difference in the reactivity between the ssPalmO-Phe and the ssPalmO-Ben can be explained by the difference in the pKa of hydroxyl groups (Figure S3, Supporting Information). The pKa of phenol is
≈10 since the phenolate anion is stabilized by the delocalization of electrons to the aromatic ring. On the other hand, the pKa of benzyl alcohol is ≈15 since the hydroxyl group is not conjugated with the aromatic ring. Thus, the reactivity of the ssPalmO-Phe was significantly higher than the ssPalmO-Ben. This observation also indicates that other esters in the LNPs (i.e., esters in ssPalm or phospholipids) would not be degraded in the HyPER reaction.

Degradation products were then identified by LC/MS. As a result, an expected compound referred to as a “head group” was identified (Figure 2c). The increase in the amount of the head group was time dependent, and well correlated with the decrease in the concentration of the parent ssPalmO-Phe. In addition, we also detected an unexpected compound which had exactly the same m/z ratio as the ssPalmO-Phe monomer (m/z 588.401). Further analysis revealed that the compound...
was a thioester compound, referred to as an “inverted head” with a free phenol group (Figure S6, Supporting Information). The inverted head compound is a possible intermediate that is produced in the HyPER reaction. In biological processes in cells, molecules with thioesters are mainly present as an S-acylated protein. The deacylation of such proteins is mediated by acyl protein thioesterases (APTs). Since the APTs catalyze the hydrolyzation of a wide range of substrates, the enzymatic degradation of the inverted head compound in cells is plausible. Moreover, since the thioester is a relatively labile structure, the inverted head compound would also be degraded to a mixture of oleic acid and head group via nonenzymatic hydrolysis.

Schematic diagram illustrating a HyPER reaction of the ssPalmO-Phe is shown in Scheme 1. The first step in the HyPER reaction is the cleavage of the disulfide bond. As shown in Figure 2 and Figure S2 (Supporting Information), this process, which is fast and reversible, would be expected to reach a plateau in the order of minutes. This estimation is in good agreement with the rate of cleavage of disulfide bonds in cytoplasm. After the first reaction, the subsequent nucleophilic attack of the thiolate to the phenol ester would occur in the intraparticle space. Since the rate of the HyPER reaction depends on the concentration of the ssPalm in the nanoparticle, as evidenced by the degradation of 4MU esters (Figure S2c,d, Supporting Information), this nucleophilic attack would be one of the rate-limiting steps. This reaction produces a highly hydrophilic conjugate composed of a head-GSH (Putative Intermediate 1) and a hydrophobic counterpart (Putative Intermediate 2). The subsequent nucleophilic attack to the Putative Intermediate 2 would result in the production of an inverted head compound as we observed. The hydrolysis of the inverted head and further reduction of the Putative Intermediate 1 in the aqueous phase would result in the generation of the final degradation products: head group, oleic acid, and oxidized form of glutathione (GSSG).

2.2. In Vitro mRNA Delivery Using the ssPalmO-Phe

2.2.1. Composition Screening of the LNPssPalmO-Phe

The mRNA-encapsulating LNPs were prepared by the ethanol dilution method. A luciferase mRNA and an EGFP mRNA were used as reporter genes. The sequence information can be found in the Supporting Information. The release of the luciferase-mRNA from the LNPssPalmO-Phe was investigated by agarose gel electrophoresis (Figure S7, Supporting Information). As shown in Figure S7a (Supporting Information), the LNPssPalmO-Ben released the mRNA only when it was dissolved with a surfactant (sodium dodecyl sulfate, SDS). On the other hand, the release of mRNA from the LNPssPalmO-Phe was triggered by treatment with a mixture of DTT and polyaspartic acid. These results confirm that when the head groups with tertiary amine and hydrophobic scaffolds are separated via a degradation reaction, the driving force to condense nucleic acid is lost and the nucleic acid is eventually released.

The lipid composition was optimized by monitoring the luciferase gene expression encoded in the mRNA (Figure 3a, Screening A). The LNPssPalmO-Phe was transfected to a CT26 mouse colon tumor cell line, and the transfection activity was monitored at 10 h after transfection. For in vitro screening,
dioleoyl-sn-glycero phosphatidylcholine (DOPC) and cholesterol were incorporated as helper lipids. The cholesterol and DOPC content was modified to 10–40% and 0–15%, respectively. In all of the compositions, DMG-PEG2000 was additionally modified at 3 mol% of the total lipid. The result of Screening A indicates that gene expression varied by 18,500-fold among the 16 lipid compositions. The transfection activity increased monotonically along with the cholesterol content. In a second screening, gene expression was monitored using a narrower range of the lipid contents (Figure 3b, Screening B): the range of the cholesterol and DOPC was set to 30–60% and 0–7.5%, respectively. The results of Screening B indicated that a cholesterol content of at least 40% was necessary for achieving a high gene expression. Moreover, the incorporation of at least 7.5% DOPC significantly improved the transfection activity. Additional screenings were also performed using other cell lines (HeLa cells and HepG2 cells) and mice (Figure S8, Supporting Information). Consistent with the results in Figure 3, the use of at least 7.5% DOPC resulted in an improved transfection efficiency. The in vitro transfection activity was elevated along with the increase of cholesterol content. On the other hand, the results of the in vivo study indicated that hepatic expression was elevated in parallel with a decrease in cholesterol content. This discrepancy between in vitro and in vivo experiments can be explained by the difference in the required stability in the extracellular environment between in vivo and in vitro conditions. In the case of in vitro transfection, the LNP ssPalmO-Phe that remained stable in the medium continuously delivered the encapsulated mRNA in an exposure time-depending manner (at least 18 h after transfection in Figure 5a). Thus, more stable LNPs with a higher cholesterol content exhibited more prominent transfection efficiency. On the other hand, a high

| Sample | O-Phe | DOPC | Chol | GM-020 |
|--------|-------|------|------|--------|
| A1     | 90    | 0    | 10   | 3      |
| A2     | 85    | 5    | 10   | 3      |
| A3     | 80    | 10   | 10   | 3      |
| A4     | 75    | 15   | 10   | 3      |
| A5     | 80    | 0    | 20   | 3      |
| A6     | 75    | 5    | 20   | 3      |
| A7     | 70    | 10   | 20   | 3      |
| A8     | 65    | 15   | 20   | 3      |
| A9     | 70    | 0    | 30   | 3      |
| A10    | 65    | 5    | 30   | 3      |
| A11    | 60    | 10   | 30   | 3      |
| A12    | 55    | 15   | 30   | 3      |
| A13    | 60    | 0    | 40   | 3      |
| A14    | 55    | 5    | 40   | 3      |
| A15    | 50    | 10   | 40   | 3      |
| A16    | 45    | 15   | 40   | 3      |
| B1 (A9) | 70 | 0 | 30 | 3 |
| B2     | 62.5  | 7.5  | 30   | 3      |
| B3 (A13)| 60 | 0 | 40 | 3 |
| B4     | 52.5  | 7.5  | 40   | 3      |
| B5     | 50    | 0    | 50   | 3      |
| B6     | 42.5  | 7.5  | 50   | 3      |
| B7     | 40    | 0    | 60   | 3      |
| B8     | 32.5  | 7.5  | 60   | 3      |

**Figure 3.** Screening of a lipid composition. a,b) Luciferase activity of the LNP ssPalmO-Phe at the first and second screening (O-Phe: ssPalmO-Phe). CT26 cells were treated with an LNP ssPalmO-Phe containing in house-prepared luciferase-mRNA. At 10 h after transfection, the luciferase activity in the lysates of the cells was measured. Each bar indicates the mean ± SD of three independent experiments. c) Size distribution of the LNP ssPalmO. The properties of the particles were obtained by dynamic light scattering (Table 1). d) A cryo-TEM image of the LNP ssPalmO-Phe that was composed of the composition B4. The TEM image of the LNP ssPalmO-Phe was acquired using a JEM-2100F field emission TEM apparatus with an accelerating voltage of 120 kV.
stability may not be required in vivo since the hepatic uptake of the LNPs generally reached completion in the order of minutes. Thus, the LNPs with a lower cholesterol content showed a higher expression. Based on the results of these screenings, we selected the composition of C11 (ssPalmO-Phe/DOPC/cholesterol = 52.5/7.5/40) for further investigations, since this composition can exhibit the high mRNA delivery efficiency both in vitro and in vivo. The self-degradation of the ssPalmO-Phe via the HyPER reaction was confirmed to proceed in the presence of the DOPC and cholesterol (Figure S9, Supporting Information). At the lipid composition with helper lipids, the release of mRNA was more moderate in comparison with that for an LNP formed with only ssPalmO-Phe (Figure S7b, Supporting Information). These results indicate that the presence of helper lipids appear to contribute to the stability of the LNPs and the controlled release of the mRNA, which resulted in a high transgene expression.

### 2.2.2. Structural Characterization of the LNPssPalms

The LNP was prepared using ssPalmO-Phe, ssPalmO-Ben, or ssPalmO. The physicochemical properties of the particles were measured by dynamic light scattering and are summarized in Table 1. All of the LNPssPalms showed a unimodal size distribution (Figure 3c). Cryo-electron microscope (Cryo-EM) imaging revealed that the LNPssPalmO-Phe formed spherical nanoparticles with electron-dense core structures. The core structures did not appear to have any visible internal structure (Figure 3d). However, a small angle X-ray scattering (SAXS) spectrum showed structural peaks for all three LNPssPalms (Figure S10, Supporting Information). Repeating distances, as estimated by the peak position, were 5–6 nm. This observation is consistent with the structural analysis of the LNP that was composed of the DLin-MC3-DMA (MC3), a component of the world’s first siRNA therapeutic, ONPATTRO.46 The repeating distance of the LNPssPalmO-Phe (ssPalmO-Phe/DOPC/Chol = 52.5/7.5/40) was comparable to the LNPssMC3 (basic composition: MC3/DSPC/Chol = 50/10/40), it is highly possible that the LNPssPalmO-Phe also forms such a disordered inverted hexagonal structure.

### Table 1. Properties of the LNPssPalms. Composition of the LNPssPalms containing luciferase-mRNA was ssPalms/DOPC/Cholesterol = 52.5/7.5/40 with an additional 3 mol% of DMG-PEG2000. Data were presented as mean ± SD (n = 6–8).

| ssPalms         | Sizea) [Å, nm] | Pdla) | Zeta potentialb) [mV] | Recovered mRNAc) [%] |
|-----------------|----------------|-------|-----------------------|---------------------|
| ssPalmO-Phe     | 79.4 ± 8.0     | 0.10 ± 0.03 | −4.0 ± 1.3            | 76.6 ± 6.7          |
| ssPalmO-Ben     | 80.9 ± 6.4     | 0.11 ± 0.02 | −5.8 ± 1.8            | 76.1 ± 7.0          |
| ssPalmO         | 86.8 ± 2.6     | 0.11 ± 0.02 | −4.6 ± 1.9            | 75.6 ± 6.3          |

a) Size, Pdl, and zeta potential were analyzed by dynamic light scattering; b) Recovered mRNA was analyzed by Ribogreen assay.

### 2.2.3. Transfection Activity of ssPalms

The LNPssPalms containing the luciferase-mRNA were transfected to mouse embryonic fibroblast (MEF) cells,46 Hepa1c1c7 mouse hepatoma cells, and HeLa human cervical cancer cells (Figure 4a). In all of the three cells, the transfection activity of the ssPalmO-Phe was significantly higher than that of the ssPalmO-Ben and ssPalmO. MEF cells were then transfected with the LNPssPalms encapsulating EGFP-encoding mRNA. The expression of the EGFP was monitored by flow cytometry and wide-field fluorescence microscopy. A flow cytometry analysis indicated that the cells that were transfected with the LNPssPalmO-Phe indicated a homogenous protein expression (Figure 4b). On the other hand, the commercially available transfection reagent (Lipofectamine messenger MAX; LFN) resulted in a heterogeneous expression of the EGFP. The EGFP positive ratio of the LNPssPalmO-Phe > LNPssPalmO-Ben > LNPssPalmO- and the LFN messenger MAX was 96.0 ± 0.8, 94.7 ± 0.5, 65.0 ± 7.3, and 25.8 ± 1.2, respectively (Figure S11a, Supporting Information). Total protein production evaluated by the averaged fluorescence indicated that the ssPalmO-Phe showed a significantly higher gene expression than the ssPalmO-Ben (Figure S11b, Supporting Information). The wide-field fluorescence microscopy findings were strongly supportive of the flow cytometry results (Figure 4c and Figure S12, Supporting Information). These results indicate that the neutral LNPs composed of the ssPalmO-Phe conferred a superior transfection efficiency and a more homogeneous gene expression in comparison to the nondegradable counterparts and the commercially available reagent.

### 2.3. Mechanism Responsible for the Improved Transfection Efficiency in LNPssPalmO-Phe

#### 2.3.1. Cellular Uptake

The cellular uptake of the fluorescence-labeled LNPs by HeLa cells was monitored at 3, 6, and 18 h post-transfection (Figure 5a). Since the rank order of the cellular uptake (ssPalmO > ssPalmO-Ben = ssPalmO-Phe) was completely different from that of luciferase expression (ssPalmO-Phe > ssPalmO-Ben > ssPalmO), the cellular uptake process cannot explain the difference in the transfection activities (Figure 4a). The same tendency was observed in the quantification of the mRNA by quantitative real-time PCR (qRT-PCR) (Figure 5c, upper). The functional availability of intracellular mRNA was then calculated by normalizing the luciferase activity by the intracellular amount of exogenous mRNA (Figure 5c, lower). The value for the LNPssPalmO-Phe was significantly higher than LNPssPalmO-Ben and LNPssPalmO. These results indicate that the ssPalmO-Phe improves intracellular processes such as endosomal escape and/or the release of mRNA compared to the ssPalmO-Ben and ssPalmO.

#### 2.3.2. Effects of HyPER Reactions on the Efficiency and Cytotoxicity

To clarify the contribution of the HyPER reaction on transfection efficiency, the luciferase activity of the LNPssPalmO-Phe was compared to that of the other LNPs using another control
material (ccPalmO-Phe). In the structure of the ccPalmO-Phe, the disulfide bond of the ssPalmO-Phe was replaced with a hydrocarbon bond (Figure 5d). Since the nucleophilic groups (thiols) are not produced in response to a reductive environment in the case of ccPalmO-Phe, degradation of the phenyl ester via the HyPER reaction would not occur. The time profile for the luciferase activity and their AUCs are shown in Figure 5e,f. The transfection activity of the ccPalmO-Phe was significantly lower than that of the ssPalmO-Phe. This result confirms that the use of a combination of a disulfide bond and a phenyl ester synergistically improved the transfection efficiency by accelerating the collapse of the LNPs. It is also noteworthy that the ssPalmO-Ben tended to exhibit a higher transfection activity than the ccPalmO-Phe. The result suggests that the reductive cleavage of the disulfide bond partially contributes to the release of mRNA although the degradation is not complete. The cytotoxicity of the ssPalmO-Phe was evaluated using Cell Counting Kit-8 (Figure S13, Supporting Information). The LNP_{ssPalmO-Phe} showed no cytotoxicity in both cell lines up to a concentration that was 64-fold higher than that used in Figure 5. On the other hand, LNP_{ccPalmO-Phe} prepared with the noncleavable ssPalm showed a significant level of toxicity in both cell lines and this toxicity was concentration-dependent. Considering these results, we concluded that the introduction of the disulfide bond to the ionizable lipid significantly suppress its toxicity.

2.3.3. Analysis of the Effect of an Aromatic Ring by a Molecular Dynamics Simulation

The functional availability of the intracellular mRNA of the ssPalmO-Ben was also significantly improved compared to
the ssPalmO (Figure 5c). As shown in Figure 6a, the hemolytic activity of the LNPssPalmO-Ben was substantially higher than LNPssPalmO at an acidic pH. Since hemolytic activity is routinely used as an index of endosomal escape,\textsuperscript{49,50} these observations prompted us to hypothesize that the insertion of the aromatic ring structure itself improves the intracellular trafficking of LNPs by enhancing the efficiency of endosomal escape.

The mechanism responsible for the aromatic ring-driven membrane destabilization was investigated from the point of view of the structural conformation and organization of the lipid molecules in the LNP using molecular dynamics simulation. Since an LNP is a supramolecular structure of lipid molecules, a dissipative particle dynamics (DPD) simulation was applied for coarse graining to simplify the calculation. The parameters used in the DPD simulation were calculated using fragment molecular orbital method (FMO, summarized in “FMO-DPD Method” (Supporting Information)).\textsuperscript{51–53} The particles produced by the FMO-DPD simulation are shown in Figure 6d. The simulated particles showed that the ssPalms were present in a “non-lamellar arrangement” in the LNPs. The head groups composed
of disulfide bonds and hydrophilic groups are assembled on the surface and inside the particles, and are particularly aligned on the surface. The structure of the individual lipid molecules ranged from linear to U-shaped rather than conventional cylindrical- or cone-shaped. The histogram of the relative angle of scaffolds is shown in Figure 6c. Two prominent differences were observed between the ssPalmO and the ssPalm-Ben: 1) π–π stacking layered structures were observed between the aromatic rings in the ssPalmO-Ben, and 2) the ssPalmO-Ben tends to form more closed conformations compared to the ssPalmO.

We previously demonstrated that the LNP ssPalms were equipped with a high fluidity and fusogenicity to the anionic lipid bilayers with respect to acidity. Since the more closed conformation of the ssPalmO-Ben is prone to have its head groups oriented outward, a conformational shift in the LNP ssPalmO-Ben may more drastically affect the interaction between the LNP and the anionic endosomal membranes compared to the LNP ssPalmO. The membrane fusion activity with a model anionic membrane of the LNP ssPalmO-Phe and LNP ssPalmO-Ben was actually higher than that of the LNP ssPalmO (Figure 6b). However, the enhanced membrane fusion cannot fully explain the significant difference in the hemolysis activity: a more dynamic change in the membrane properties of erythrocytes on the addition of the LNP ssPalmO-Ben would contribute to the extent of hemolysis. The formation of the π–π stacked layered structures indicates that the mutual interaction between the ssPalmO-Ben molecules was higher than that of the ssPalmO molecules. It is possible that the ssPalmO-Ben that was transferred on the lipid bilayer on erythrocytes might assemble into a de novo nonlamellar domain on it. The formation of a hydrophobic nonlamellar domain per se might be a driving force for membrane destabilization, and also might trigger further lipid mixing between LNPs and erythrocytes, thus accelerating the extent of membrane destabilization.

Figure 6. Effects of the aromatic ring in the scaffold. a) Hemolysis activity of the LNP ssPalms. Mouse erythrocytes were incubated with LNP ssPalms at the pH 5.5, 6.5, or 7.4. The amount of leaked hemoglobin was quantified based on the absorbance at 545 nm. b) Membrane fusion activity with anionic liposomes. DiA/DiD-labeled LNP (colabeled with 0.5 mol% DiD and DiA (Figure S14, Supporting Information)) was incubated with DOPS/DOPC anionic liposomes at pH 5.5, 6.5, or 7.4. The FRET cancellation was analyzed as described in methods section. c) Distribution of intramolecular angles of two hydrophobic scaffolds of ssPalms. d) Particle surfaces and sectional drawing near the center. Bottom figures are the shapes of some of the lipid molecules. Blue, orange, and cream colors represent segments D-F (S-S, piperidine, ester), G (phenyl), and others, respectively. The segmentation of molecule and definition of the intramolecular angle is shown in Figure S14 (Supporting Information) in “FMO-DPD Method” (Supporting Information).
The cellular uptake of the LNP_{ssPalm} was completely inhibited when the incubation was conducted on ice (data not shown). This result indicates that the LNP_{ssPalm} are internalized by cells by an energy-dependent endocytosis pathway. A microscopic analysis of the fluorescent-labeled LNPs taken up via endocytosis was performed at 3, 6, and 18 h after transfection by confocal laser-scanning microscopy (CLSM, Figure 7a). The cytoplasm of the HeLa cells was stained by calcein-AM. In all of the ssPalms, the fluorescence of DiD was detected as punctate forms at 3 h after the transfection. At 6 and 18 h after the transfection, the DiD signals were still detected as dots in the case of the LNP_{ssPalmO-Ben} and LNP_{ssPalmO}. Thus, the LNPs still exited as a particle and/or were trapped in endosomes/lysosomes. In contrast, the DiD signals were widely distributed in whole cells in the case of the LNP_{ssPalmO-Phe}. This indicates that the DiD released from the LNP_{ssPalmO-Phe} by the collapse of particles was...
widely distributed to the whole cells by binding to cellular proteins or membranes.

The intracellular collapse of the LNPs was also monitored by in-cell Förster resonance energy transfer (FRET) cancellation using flow cytometry. The LNP_{ssPalm} was labeled with a pair of fluorescent dyes. As shown in Figure S14 (Supporting Information), the combination of DiA (donor: 488/572 nm) and DiD (acceptor: 645/676 nm) was a useful FRET pair, since the fluorescence intensity of DiA was suppressed to \( \approx 5\% \) by FRET when the LNP was labeled with both dyes. Since the FRET efficiency depends on the relative distance between the DiA and DiD, the fluorescence of DiA would recover when these dyes are separated from each other. In fact, the fluorescence of DiA recovered almost completely on treatment with a surfactant (Triton-X100, Figure S14, Supporting Information): the fluorescence of DiA was nearly the same as that for the particle labeled with DiA only. The DiA/DiD-labeled LNPs were transfected to the HeLa cells. DiA-labeled LNPs were also transfected as control samples. At 6 or 21 h after the transfection, the cells were collected, and the intensities of DiA fluorescence in each cell were monitored by FACS. Theoretically, less DiA fluorescence should be detected when the DiA/DiD-labeled LNPs remained stable in the cells. In contrast, in-cell FRET cancellation of the DiA/DiD-labeled LNPs that is detected in terms of the recovery of the DiA fluorescence to the level comparable to that for the DiA-labeled LNP-transfected cells reflected the collapse of the LNPs or their fusion with biomembranes in cells. Representative FACs charts are shown in Figure 7b. Intracellular FRET cancellation was calculated by dividing the geometrical mean of the fluorescence (Ex/Em = 488/572 nm) of the DiA/DiD-labeled LNPs by that of the DiA-labeled LNPs. The in-cell FRET cancelation data also support our conclusion that the lipid components of the LNP_{ssPalmO-Phe} are dissociated from each other after the particles collapse (Figure 7c), although the contributions of degradation in endo/lysosomes, fusion with endosomal membranes, and the intracellular collapse of the LNPs via HyPER reaction cannot be distinguished from one another.

Considering these results, we conclude that the introduction of disulfide bonds and phenyl esters enhances the intracellular delivery of loaded mRNA by two mechanisms: 1) the “Effects of the HyPER reactions”: Intra-particle reactions (HyPER) based on the combination of disulfide bonds and phenyl esters promote the intracellular release of mRNA, and 2) “Effects of aromatic rings”: insertion of an aromatic ring into the hydrophobic scaffold of ionizable lipids enhances membrane destabilization activity. Since the overall gene expression was rate-limited by sequential processes including cellular uptake, endosomal escape, the release of mRNA, and translation, the efficiency of each step would contribute to the overall improvement in gene expression (47.5-fold) individually. The “Effects of the HyPER reactions” and “Effects of aromatic rings” were evaluated by comparing the activities of the “ssPalmO-Phe versus the ssPalmO-Benz” and the “ssPalmO-Phe versus the ssPalmO,” respectively. Figure 5c shows that the contribution of the “Effects of aromatic rings” and the “Effects of the HyPER reactions” were 18.5-fold and 2.57-fold, respectively. While the contribution of the “Effects of HyPER reaction” was relatively smaller compared to the “Effects of aromatic rings,” partly because the original ssPalm per se is bio-degradable with the aid of disulfide bonding, it is worthwhile to emphasize that HyPER reaction-triggered self-degradation is a quite important event in terms of maximizing protein production with high reproducibility in various types of applications (Figures 4a, 5f, and 8a, and Figure S11b, Supporting Information). Since the intracellular amount of the mRNA delivered by the LNP_{ssPalmO-Phe} was only 0.32% of the transfected dose (Figure 5c), improving the cellular uptake process by combining with a targeting ligand would further increase the mRNA transfection efficiency in vivo and in vitro.

2.4. In Vivo mRNA Delivery

2.4.1. In Vivo Liver Delivery of the mRNA

LNPs encapsulating a mouse erythropoietin (mEPO)-mRNA were administrated to a BALB/c mouse via the tail vein. As a control, the LNP was prepared with a composition of MC3/DSPC/cholesterol/DMG-PEG_{5000} = 50/10/38.5/1.5.[10,47] The properties of the in vivo applied particles are summarized in Table 2. The time profile for the mEPO concentration in blood and the calculated AUCs are shown as Figure 8a. The gene expression of the LNP_{ssPalmO-Phe} was significantly higher than the other LNPs. An in vivo imaging system (IVIS) analysis revealed that the mRNA was dominantly transfected to the liver as evidenced by monitoring luciferase-encoding mRNA (Figure 8b and Figure S15, Supporting Information). Furthermore, the LNP_{ssPalmO-Phe} introduced a more than tenfold higher gene expression compared to the commercially available Invivofectamine 3.0 (Figure 8c,d).

2.4.2. Repeated-Dosing of the LNP_{ssPalmO-Phe} Prepared Using Microfluidic Device

Since mRNA does not have the ability to self-replicate, its expression is inevitably transient. To maintain the protein expression, repeated doses are needed.[1,2,22] To investigate the feasibility of the LNP_{ssPalmO-Phe} for use in a repeat-dose regime, LNPs encapsulating human EPO-encoding mRNA with chemically modified nucleotides (5-methoxyuridine (5moU) or pseudouridine (PsU), TriLink BioTechnologies) were prepared. The LNPs were administrated five times at weekly intervals (Figure 9a,b). The blood concentration of the hEPO was monitored at before (Day-1), at 6 h (Day0), and at 24 h after the administration (Day+1). The LNP_{ssPalmO-Phe} was prepared using a microfluidic device (NanoAssemblr). The properties of the particles are summarized in Table 3. As a result, the LNP_{ssPalmO-Phe} successfully introduced the hEPO after each administration. At 6 d after the administration, the blood concentration of the hEPO was below the detection limits. This finding serves to confirm that the gene expression caused by the LNP_{ssPalmO-Phe} is transient and that frequent administration is feasible.

2.4.3. Genome Editing Using CRISPR/Cas9 System

Promising applications of the mRNA delivery system include genome editing, since the effect of editing continued as long as the cells remained alive. This technology is thus one of the
potent solutions for overcoming the disadvantage of mRNA delivery, where the expression of the protein is transient. For this purpose, commercially available Cas9 mRNA with a 5moU modification was encapsulated in the LNP_{ssPalmO-Phe} with a chemically modified sgRNA that targets the mouse transthyretin (TTR) gene.\textsuperscript{[20]} Detailed information regarding the methods and physicochemical properties of the LNPs are summarized in Sections S2 and S3 (Supporting Information). Two injections of the LNPs containing both mRNA and sgRNA (Figure 9d) resulted in a 95% decrease in the serum TTR concentration (Figure 9e). Electrophoresis-based mutation assays indicated that the 55% of the TTR-encoding genome were edited (Figure 9f,g).

2.4.4. Acute Liver Toxicity Evaluation

As described in the in vivo delivery section, the main target of the LNP_{ssPalmO-Phe} is the liver. To investigate effects on the organ that expresses mRNA, acute liver toxicity was evaluated. Details of the acute liver toxicity evaluation including information

| Lipids          | Size$^a$ [d, nm] | PdI$^b$ | Zeta potential$^c$ [mV] | Recovered mRNA$^c$ [%] |
|-----------------|------------------|---------|-------------------------|------------------------|
| ssPalmO-Phe     | 86.5 ± 4.0       | 0.09 ± 0.001 | −3.0 ± 0.6             | 79.0 ± 2.9             |
| ssPalmO-Ben     | 84.8 ± 9.1       | 0.09 ± 0.02  | −4.6 ± 0.6              | 82.4 ± 6.0             |
| ssPalmO         | 88.2 ± 7.2       | 0.10 ± 0.01  | −4.9 ± 0.7              | 72.3 ± 1.0             |
| MC3             | 55.8 ± 6.6       | 0.10 ± 0.01  | −5.2 ± 0.2              | 78.6 ± 5.6             |

$^a$Size, PdI, and zeta potential were analyzed by dynamic light scattering.

$^b$Recovered mRNA was analyzed by Ribogreen assay.

Figure 8. In vivo function. a) Time-dependent production of mouse EPO. BALB/c mice (female, 6 weeks) were administrated with the LNP_{ssPalmO} or the LNP_{MC3} containing in-house prepared mouse EPO-mRNA via the tail vein (0.05 mg kg$^{-1}$) (O-Phe: ssPalmO-Phe, O-Ben: ssPalmO-Ben). Concentration of EPO in serum was evaluated by ELISA. Each bar indicates mean ± SD (n = 3). Statistical analyses were performed by one-way ANOVA followed by Bonferroni’s multicomparison test (**p < 0.01). b) In vivo luciferase assay using an In Vivo Imaging System (IVIS). At 3 h after administration of the LNP containing luciferase-encoding mRNA, the mice were injected with α-luciferin potassium PBS(−) solution (3 mg/200 µL/head). At 15 min after the injection of the luciferin solution, the luminescence was measured by IVIS. c) Comparison with Invivofectamine. The LNPs_{ssPalmO-Phe} or Invivofecamine 3.0 containing luciferase-mRNA was administrated at a dose of 0.1 mg kg$^{-1}$. d) Comparison of transgene expression. Luciferase activity was calculated from the images in (c). Each bar indicates the mean ± SD (n = 3). Statistical analysis was performed by the Student’s t-test.
regarding the approval of experiments by the ethics committee can be found in the Supporting Information. The effects of a single administration of the ssPalmO-Phe were evaluated using an empty LNP ssPalmO-Phe (without mRNA). The LNPs were administrated to Sprague-Dawley SPF rats [Crl:CD(SD), five males/group, six weeks of age at the start of administration], and hematology and blood chemistry were investigated 1 d postadministration. The results indicate that the maximum dose without observed liver toxicity of the intravenously administrated LNP ssPalmO-Phe was between 70 and 175 mg kg\(^{-1}\) of total lipids (corresponding to 49.6–124 mg kg\(^{-1}\) ssPalmO-Phe). Liver toxicity was also evaluated using BALB/c mice. The AST/ALT ratio increased starting at a dose of 350 mg kg\(^{-1}\) total lipids (248 mg kg\(^{-1}\) ssPalmO-Phe, Figure S16, Supporting Information). The liver injury of the ssPalmO-Phe was less than that of the MC3. The less toxic nature of the ssPalm against the liver can be attributed to the biodegradability of the disulfide bond (Figure S4, Supporting Information). This conclusion was
also supported by the fact that L319, a biodegradable derivative of the MC3 that contained an ester moiety in the hydrophobic scaffold, was less toxic.[54] The other components of the LNP_{ssPalm} (i.e., DOPC and cholesterol) are not degraded during the HyPER reaction. These lipids are probably cleared from the body after entering the intrinsic lipid metabolic pathways.[55]

In the case of neutral particles such as the LNP_{ssPalmO-Phe}, apolipoprotein E dependent liver accumulation is primarily assumed for their pharmacokinetics.[56,57] The results of in vivo delivery experiments are consistent with this assumption (Figure 8b,c). However, the distribution of mRNA is highly dependent on the molecular structure of the ionizable materials being used[58] and/or the composition of the formulation.[59,60]

Thus, the possibility that the LNP_{ssPalmO-Phe} could reach unintended targets in the body and affect their function cannot be completely ruled out. This point should also be carefully investigated in the case of genome editing, since the changes in the genome sequence probably have a permanent effect on cell function. Comprehensive identification of the expression site[60] and assessment of acute/chronic toxic effects on the whole body would be prerequisite for further applications.

3. Conclusion

The use of a combination of a disulfide bond and a phenol ester in one molecule can be used for the self-degradation of ionizable lipids via the HyPER reaction. The degradation of the ssPalmO-Phe contributes to the active collapse and intracellular release of the mRNA, which was found to improve the transfection efficiency, both in vitro and in vivo. The insertion of the aromatic ring also contributed to the improved endosomal escape. Since the intracellular trafficking is a sequential process that includes cellular uptake, endosomal escape, the release of mRNA, and translation, the improvement in each process synergistically enhances the gene expression.

4. Experimental Section

Details of the experiments were summarized in the Supporting Information.

Animal Experiments: C57BL/6J mice (male or female, six weeks), BALB/c mice (male or female, six weeks), and ICR mice (male, six weeks) were purchased from Japan SLC, Inc. (Shizuoka, Japan). The experimental protocols were reviewed and approved by the Chiba University Animal Care Committee in accordance with the “Guide for Care and Use of Laboratory Animals.”

Molecular Dynamics Simulation: Detailed information for the FMO-DPD simulation[51–53] can be found in “FMO-DPD Method” (Supporting Information).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

Chiba University and the NOF CORPORATION hold a pending patent on the ssPalm chemicals. In addition, H.A. received research funding from the NOF CORPORATION.

Keywords

ionizable lipid, messenger RNA, nanoemulsion, self-degradability

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