# Evaluation and Classification of Intraparticle Structure of Ready-to-Use Lipid Nanoparticles Encapsulating Messenger RNA

Messenger RNA synthesized by *in vitro* transcription reaction (IVT-mRNA) is an important modality for gene therapy. Since the IVT-mRNA is susceptible to the enzymatic degradation in our body, the development of drug delivery system (DDS) is prerequisite for its therapeutic use. Lipid nanoparticles (LNPs) are promising DDS for the delivery of IVT-mRNA. However, the requirement of expensive equipment and know-how has been an entry barrier for the research field of mRNA-LNPs. In this study, we developed user-friendly Ready-to-Use type LNPs formulation (LNPs(RtoU)). Intraparticle structure of this formulation was compared to that of conventional LNPs using small angle X-ray scattering (SAXS).

Messenger RNA synthesized by *in vitro* transcription reaction (IVT-mRNA) has attracted attentions in the field of pharmaceutical sciences. Since the IVT-mRNA can introduce any desired proteins to our body, the IVT-mRNA is expected to realize various therapeutic modalities. However, the IVT-mRNA is highly vulnerable to the enzymatic degradation. Therefore, therapeutic use of the IVT-mRNA requires the development of drug delivery systems (DDS). Lipid nanoparticles (LNPs) containing pH-responsive ionizable lipid are promising DDS for the nucleic acid therapeutics; mRNA-LNPs with the IVT-mRNA coding an antigen protein of SARS-CoV2 has been used as an RNA vaccine worldwide.

mRNA-LNPs are nano-size assemblies of hydrophilic IVT-mRNA molecules and hydrophobic lipid molecules. As a preparation method for the mRNA-LNPs, alcohol dilution method has been generally used (Fig. 1(a)). In this method, mRNA dissolved in acidic buffer and lipids dissolved in ethanol were mixed using the microfluidic mixier. As the solubility of lipids decreases, the negatively charged IVT-mRNA and the positively charged pH-responsive ionizable lipids co-precipitate as the mRNA-LNP. While this alcohol dilution method is well-established, however, there is a huge hurdle for life science researchers to prepare the mRNA-LNP because of its requirement of expensive equipment and know-how.

In the present study, we developed a Ready-to-Use type formulation for post-encapsulation of IVT-mRNA (mRNA-LNP(RtoU)) (Fig. 1(b)) [1]. In this formulation, a solution of IVT-mRNA was added to the freeze-dried empty-LNPs and then incubated at 75-95 ºC. By adjusting the pH of formulation to pH6.0 when rehydrated, IVT-mRNA can be encapsulated inside the LNP by electrostatic interaction. Differential scanning calorimetry (DSC) analysis revealed that an irreversible endothermic peak corresponding to the post-encapsulation process appeared at 15-30 °C. In-situ measurements of the sample temperature showed that the encapsulation efficiency reached a plateau after the sample temperature exceeded 72.6 °C.

The key question was whether mRNA-LNPs prepared by conventional microfluidic mixing (mRNA-LNPs(MF)) and mRNA-LNPs(RtoU) prepared by the post-encapsulation have an equivalent intraparticle structure. We compared the intraparticle structures of these nanoparticles by using small angle X-ray scattering (beamline BL-10C, BL-6A) (Fig. 2). The parameters investigated were <1> the method used to prepare LNPs (RtoU or MF), <2> the pH of the LNPs(RtoU) (pH5.5 or pH6.0), <3> the presence of IVT-mRNA and its chemical modification (empty, uridine-type IVT-mRNA (U), or N1-methylpseudorudine modified IVT-mRNA (m1Ψ)), and <4> the use of heating. The diffraction images from these LNPs were analyzed by means of the SAngler software (ver. 2.1.39) [2] and then processed by calculating the slope at each scattering wavenumber q (q-Slope plots). These q-Slope plots were then analyzed by a principal component analysis and subsequent Ward's clustering analysis. The particles could be statistically clustered into four groups. The most important observation is that mRNA-LNPs(RtoU) has similar intraparticle structures to conventional mRNA-LNPs(MF) (Cluster 2). To extract structural information that were reflected in the PCA, the eigenvectors of PC1 and PC2 were focused on. PC1 included the information of "the presence or absence of heating", and the information was reflected in the regions around q = 0.185 Å$^{-1}$ and q = 0.369 Å$^{-1}$. The peaks in these regions, which corresponded to Bragg's distances (d = 2π/q) of 33.9 Å and 17.0 Å, was derived from cholesteryl monohydrate crystals [3]. These peaks disappeared after temperature increase over 55 °C. PC2 included the information of "the presence or absence of IVT-mRNA", and the information was reflected in the region around q = 0.097 Å$^{-1}$ which corresponded to a Bragg's distance of 64.7 Å. The peaks around q = 0.1 Å$^{-1}$, expected to be a complex of lipids and nucleic acids, were also reported in other literatures using siRNA-LNPs [4] and pDNA lipoplexes [5] as well as other mRNA-LNPs [3, 6].

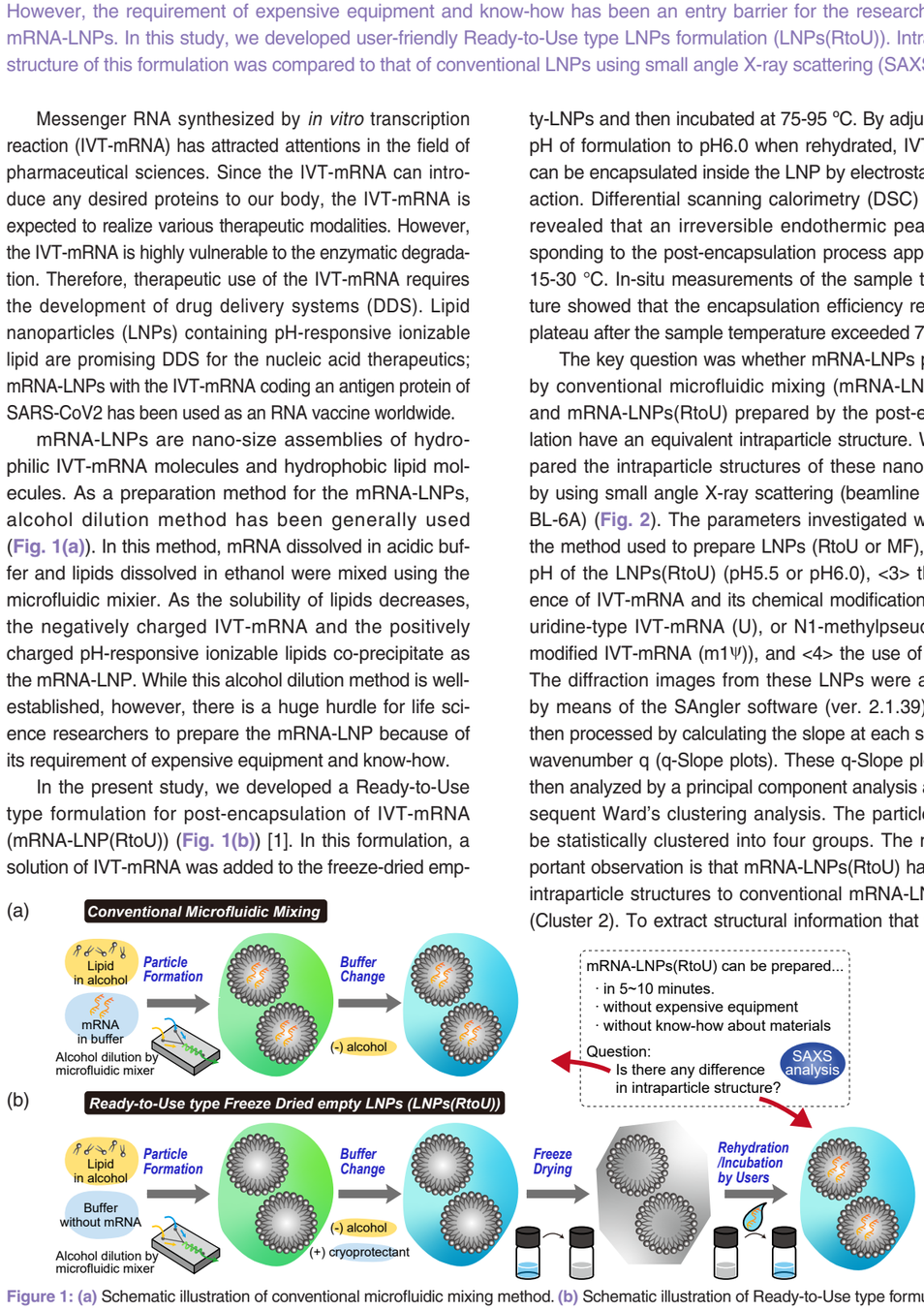


**Figure 1: (a)** Schematic illustration of conventional microfluidic mixing method. **(b)** Schematic illustration of Ready-to-Use type formulation.

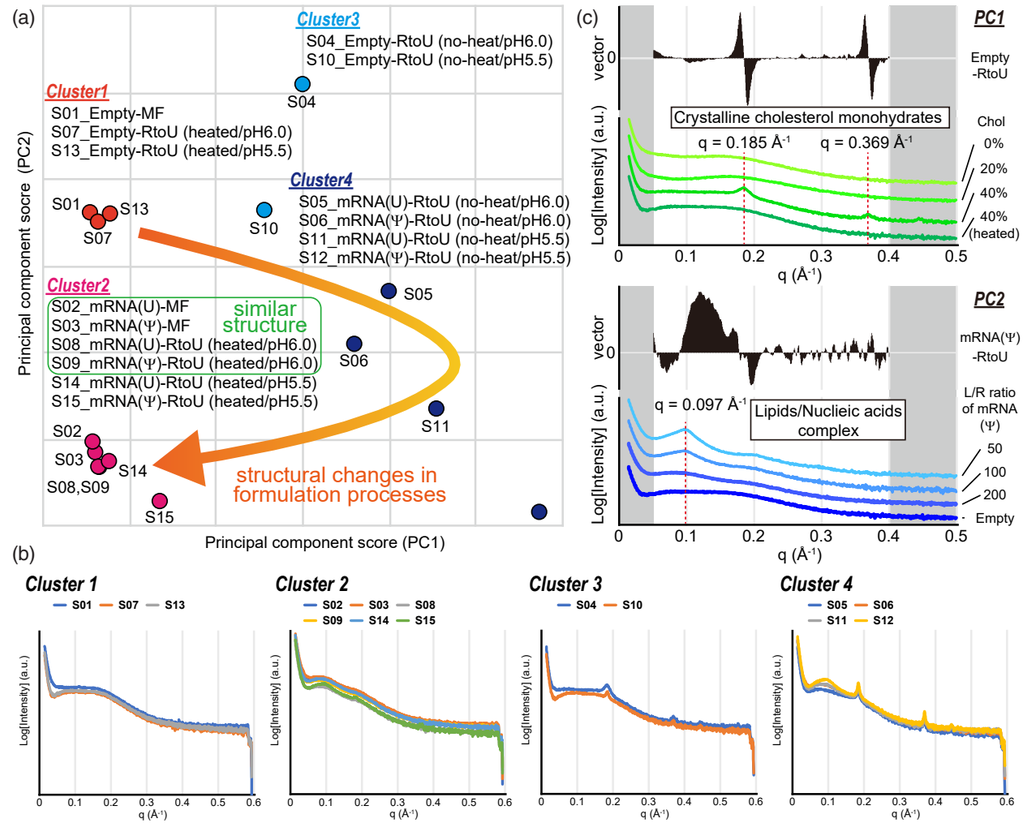


**Figure 2: (a)** Cluster analysis of LNPs based on their intraparticle structure. The SAXS charts obtained from each LNP are processed to q-Slope plots. The q-Slope plots were then analyzed by PCA and subsequent Ward's clustering. **(b)** SAXS charts of LNPs which were categorized to each cluster. **(c)** Analysis of eigenvectors for principal component score.

The hypothesized mechanism of the post-encapsulation is summarized as follows; During freeze-drying, cholesterols form crystalline cholesteryl monohydrate. After rehydration, the irreversible post-encapsulation starts at 15-30 °C. This post-encapsulation is completed at approximately 75 °C. In parallel, the crystalline cholesteryl monohydrates are dissolved into the LNPs at temperature above 55 °C. After these structural changes, the mRNA-LNP(RtoU) forms similar structure to the conventional mRNA-LNP(MF). Therefore, the LNP(RtoU) is an alternative formulation that can be used with simple procedure.

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## BEAMLINES

BL-10C and BL-6A

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