bypassing early endosomes. Surprisingly, siRNA that dissociated from the LNP was exocytosed to the extracellular milieu from macropinosomes or late endosomes (**Fig. 1**). Loss of siRNA in this manner accounted for 70% of the uptake dose 24 h after transfection. This previously unknown mechanism that compromises siRNA delivery by this LNP should provide an opportunity to develop approaches for retaining siRNA inside cells.

In the second paper, Gilleron *et al.*[3] studied LNPs loaded with siRNA that was labeled by either fluorescent dyes or gold nanoparticles and analyzed trafficking with quantitative fluorescence imaging and electron microscopy in different cell types *in vitro* and in mouse liver. Their LNPs, based on a pH-sensitive, ionizable lipid coated with Apolipoprotein E, have been successfully tested in various species, including nonhuman primates[7,8]. In cell culture and after intravenous injection, the LNPs were found to enter hepatocytes through both macropinocytosis and clathrin-mediated endocytosis (**Fig. 1**). Although the authors suggested that clathrin-mediated endocytosis is a prerequisite for activation of macropinocytosis (as is the case in adenovirus-mediated infection), most of the siRNA entered by macropinocytosis.

The use of electron microscopy to detect intracellular, gold-conjugated siRNA substantially elevated the sensitivity of these experiments. Conventional fluorescence microscopy is often unable to detect minor intracellular populations of siRNA. Gilleron *et al.*[3] determined that only a minor fraction (1–2%) of internalized siRNA could escape from the endocytic system. They also identified the moderately acidic, early endosomal compartment as the main site of release of siRNAs into the cytosol (**Fig. 1**). This observation indicates that escape from the endosome is the critical, rate-limiting step in the delivery of siRNA with this LNP and that methods that increased retention in early endosomes might improve endosomal escape and overall delivery efficiency.

In both studies, the LNPs were found to enter cells primarily by macropinocytosis. With the LNP used by Sahay *et al.*[2], ~70% of internalized siRNA ended up in the extracellular milieu, whereas Gilleron *et al.*[3] did not observe any exocytosis of siRNA. An important determinant of delivery is the efficiency of siRNA escape from endosomes into the cytosol. Gilleron *et al.*[3] estimated that <2% of the siRNA escaped from early endosomes, suggesting that optimization efforts should be directed to this issue. Sahay *et al.*[2] did not address this question.

The differences between the findings in the two papers are likely due to differences in the cell types investigated and in the properties of the lipid or lipidoid used in the LNPs. The major difference in the LNPs was in the cationic lipid. Apparently, the structure of the cationic lipid governs the intracellular trafficking pathways of the siRNA carried by the vehicles.

Although the LNPs in the two studies are composed of different lipids, they are similar in that both were derived through a screening process designed to find simple materials that simultaneously solve all delivery challenges, from ensuring siRNA stability to finding the target tissue and facilitating cytoplasmic delivery. That this will lead to materials that perform well on average but do not excel in any particular step of the delivery process is widely suspected—and nicely illustrated by the data of Sahay *et al.*[2]. Seventy percent of siRNA delivered by their C12-200 LNP was exocytosed to the extracellular milieu. However, there were other lipidoids that performed better than C12-200. For example, lipidoids with longer chains resulted in higher intracellular retention of the internalized siRNA but were not selected as the best for overall delivery efficiency *in vivo* in the initial screen[2].

The problem with this screening strategy is its reliance on a single material. A single lipid or lipidoid is hardly sufficient to solve all the challenges imposed by a cell, or even a single subcellular compartment. At the organismal level, additional delivery issues, such as serum stability, evasion of the reticuloendothelial system and hepatocyte uptake, also demand effective solutions. An alternative approach is to formulate multiple materials with different unique properties, such as facilitating cell uptake or enhancing endosomal escape, into one delivery vehicle. Such combinations of materials can synergistically achieve efficient siRNA delivery[9]. Another approach is to employ a modular design in the development of nanoparticles with defined structures. Good examples of this modular design are the multiple-envelope nanodevices[10] and lipid-calcium-phosphate nanoparticles[11]. In these nanoparticles, different modules are designed to overcome different extracellular and intracellular barriers. Although the development of such nanoparticles is still at an early stage, they represent an important example of rational design to achieve siRNA delivery.

Nonetheless, the papers by Sahay *et al.*[2] and Gilleron *et al.*[3] are notable in that they examine the delivery vehicles that are furthest along in clinical development. Quantitative models of trafficking are clearly important for the rational design of nanoparticles, and the two studies can serve as a blueprint for how to analyze similar processes for other macromolecular drug delivery technologies. The challenge now is to use the information to develop better siRNA therapeutics.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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