The benefits of emerging alternatives to PEG for lipid nanoparticle RNA delivery systems

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Introduction

For several years, significant efforts have been devoted to developing efficient non-viral vectors for nucleic acids delivery, including mRNA and siRNA. This approach has been successful, as evidenced by the FDA approval of four lipid nanoparticle (LNP)-based RNA therapeutics since 2018: Onpattro®, Comirnaty®, Spikevax®, and mResvia®. To achieve that, work has been done on the LNP formulation, in particular on the ionizable lipid and lipid-polyethylene glycol (PEG). In addition to these developments, increasing efforts are being made to identifying alternatives to PEG. While PEG has been widely used in various applications, it has also been extensively studied, and concerns about its use emerged. Two major drawbacks associated with PEGylation in nanomedicines have been well documented. The so-called *PEG dilemma* encompasses the steric barrier conferred by PEG, which hampers vector transfection efficiency, and its immunogenicity, which leads to anti-PEG antibody production and particle accelerated blood clearance (ABC effect). This dilemma is then responsible for compromised therapeutic efficacy, particularly upon repeated administrations [1].

Therefore, alternative polymers have been designed to replace PEG. They should ideally confer similar benefits in terms of particle stability and protection, stealth properties, while limiting PEG drawbacks. Among these alternatives, some have been used to modify lipid-based nanoparticles for RNA delivery and will be described below.

While the PEG issues are well known and alternatives actively being developed, it is worth questioning why all four approved RNA-LNP formulations still contain PEG, and why its replacement in clinical applications remains challenging. This raises the question of whether there is a real need for PEG alternatives.

Should PEG be avoided in lipid-based RNA vectors?

Although PEG is generally considered poorly immunogenic, its association with nanocarriers such as PEGylated drugs leads to immunogenicity and antibody production [2,3]. Furthermore, due to its widespread use in cosmetics, food and other applications, a high prevalence of pre-existing antibody has been observed in the population [4], with a higher incidence in women, likely due to exposure to cosmetics [3,5].

While these antibodies may lead to cross-reaction and fast clearance of other PEGylated therapeutics, they also have been linked to hypersensitivity reactions. One of the mechanisms involved is complement activation, which has been shown to compromise the integrity of PEGylated liposomes and LNPs, leading to premature cargo release or

exposure [2,6,7], and possibly causing complement activation-related pseudo-allergy (CARPA) [8]. While complement activation can enhance the immune response in the context of intramuscular (IM) administration of LNP vaccines [2], it may have adverse effects in other therapeutic applications.

Recent studies have investigated the impact of PEG antibodies on PEGylated LNP. In mice stimulated with intravenous (IV) PEGylated liposomes, PEG antibodies were found to reduce the circulation time and alter the distribution of IM LNP-mRNA vaccines, but surprisingly did not affect their vaccination efficacy [3]. While the low impact of antibodies on the efficacy of IM-administered LNP vaccines appears favorable, the effects are expected to be significantly different for IV-administered LNPs used in other therapeutic applications, especially in cases of specific tissue targeting. Furthermore, intravenous injection of high doses of LNP in stimulated mice resulted in lethality within 1 hour [3], raising safety concerns about the use of high-dose IV injection of LNP in patient with anti-PEG antibodies. Another study reported an increase in PEG IgG and IgM levels following SARS-Cov-2 mRNA vaccination, especially after Spikevax® vaccination (probably due to the higher PEG content per dose) [5,9]. This may enhance the interaction between other LNPs and blood immune cells (particularly phagocytes), which should be considered in the context of IV administration. Similarly, while PEG antibodies did not affect the SARS-Cov-2 neutralizing antibody response after vaccination, this phenomenon should be monitored after additional booster doses [5].

Therefore, while some of the PEG drawbacks do not seem to hinder clinical development, such as the IM LNP formulation, factors such as the boost in PEG antibodies due to COVID-19 vaccination, the high prevalence of pre-existing antibodies in women, the higher reactogenicity after IV LNP administration, and the lack of data on the impact of antibodies on IV therapeutic LNP warrant greater caution, and underscore the need for alternatives for application beyond vaccination. However, there remain insufficient human evidence to conclusively establish a link between anti-PEG antibodies and anaphylaxis [10].

Which alternatives for lipid-based RNA vectors?

To date, several alternatives for lipid-based RNA vectors have emerged: polysarcosine (pSar), poly(N-vinyl pyrrolidone) (PNVP), poly(N-methyl-N-vinylacetamide) (PNMVA), polyoxazoline, polyoligo(ethylene glycol) methyl ether methacrylate (POEGMA), poly(N-(2-hydroxypropyl) methacrylamide) (PHPMA), poly(N,N-dimethylacrylamide) (PDMA), ...

In the context of LNP formulation, PEG plays a crucial role in stabilizing particle size, as it is currently not possible to produce LNP without PEG. Therefore, for polymers to be considered suitable alternatives, they should be first able to stabilize LNP size. Depending on both lipid and polymer chain length, but also the content, pSar met this requirement [8,11], as did PNMVA [12], POEGMA, PHPMA, PDMA [13], or poly(2-methyl-2-oxazoline) (PMOZ) [14].

Given the dilemma described with PEG, alternatives should have a minimal impact on nanoparticle transfection. Recent studies have shown that pSar mRNA-LNPs showed lower luciferase expression *in vitro* than PEG-LNPs, but substitution of the ionizable lipid promoted comparable or even higher expression *in vivo* [8]. Other studies have shown improved transfection efficiency both *in vitro* and *in vivo*, with the results being highly

dependent on the conjugated lipid [11,15], demonstrating that dilemma can vary depending on the formulation as well as the target cells. DSPE-PNMVA$_{24}$, included in both siRNA-lipoplexes and LNPs, showed reduced dilemma compared to DSPE-PEG, even in serum-containing medium, with more than 80% GFP fluorescence inhibition whereas PEG-LNPs demonstrated ~ 65% and 45% fluorescence extinction in serum-free and serum-containing conditions, respectively [12]. Similarly, mRNA POEGMA- and PDMA-LNP showed higher luciferase protein expression *in vivo* compared to PEG-LNP. In contrast, the PMOZ content had to be reduced to 1% to improve LNP transfection efficiency *in vitro* and increase immunostimulatory potential compared to 1.5% PEG-LNPs. Interestingly, while PMOZ-LNPs induced a lower antigen-specific T-cell response, they elicit a higher antibody response *in vivo* [14].

Finally, to achieve prolonged blood circulation of the vectors, immunogenicity and ABC effect should be minimized. Several alternatives are currently under investigation, including POEGMA-DSG, PHPMA-DSG, PDMA-DSG [13], and some showed promising preliminary results. Reduced immunogenicity has been observed following IV injection of DSPE-PNMVA$_{24}$ or DSPE-PNVP$_{30}$ lipoplexes in mice, with lower levels of polymer antibodies compared to PEG antibodies, and no inflammation-related response (assessed by pro-inflammatory cytokine production) [12,16]. These results should be confirmed with LNP formulation. Notably, previous studies showed no ABC effect for PNVP-liposomes [17], although reduced transfection efficiency was observed with PNVP-lipoplexes [16]. Regarding the pSar-LNP, studies have shown its safe use, with lower pro-inflammatory cytokine secretion and reduced complement activation compared to PEG-LNP [8].

Other advantages must also be taken into account when developing alternatives to PEG, such as their production method. Recently, PNMVA, PNVP, POEGMA, PHPMA and PDMA were produced using reversible addition fragmentation chain transfer (RAFT) polymerization, a versatile and easy to perform technique for well-defined polymers, compared to the LIP method used for PEG production [13]. Additionally, pSar and poly(2-oxazoline)s can be synthetized by ROP (ring-opening polymerization) approach, as well described elsewhere [7].

While PEG effectively limits protein corona formation around nanoparticles, this shielding remains partial, and a corona can still form [18]. Nevertheless, DSPE-PNVP and -PNMVA derivatives proved effective in protecting lipoplexes and LNPs from corona formation in serum-containing medium [12,16]. In some applications, such as liver targeting, corona formation can even be advantageous, as illustrated by the development of Onpattro®. As such, it may be worth considering alternatives that deliberately modulate the corona formation and composition. Interestingly, alongside the exploration of alternatives, stealth unPEGylated liposomes have been developed, exploiting DNA electric charge to form proteoDNAsome covered with an opsonin-deficient corona [18].

Overall, the development of a library of alternative polymers would be valuable to enable their application across various contexts, allowing for the modulation of nanoparticle properties and the targeting to specific tissues or cell types.

Conclusion

Recent studies have shown that complete replacement of PEG with an alternative can produce LNP with intended properties, while maintaining or even improving RNA delivery. Therefore, the decision to replace PEG for RNA delivery may depend on multiple factors. Regarding the route of administration, intravenous delivery appears to be the most challenging due to reactogenicity associated with PEG antibodies, which may therefore require the use of less immunogenic alternatives. This may be even more relevant given the increase in antibodies observed following COVID-19 vaccinations and the potential need for future administrations. Intravenous administration of therapeutic LNPs (e.g., to treat cancer or genetic diseases) should then be considered differently from intramuscular administrations of vaccines, especially when repeated administrations are required. For other administration routes, such as the pulmonary route, few studies are available on PEG replacement and mucopenetrative properties [19]. Moreover, the approval of these new alternatives by regulatory authorities remains a challenge. Some potential approaches have been proposed, such as the use of biosimilar-like regulatory guidelines and funding for large-scale longitudinal studies on polymer immunogenicity [6].

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