COMMENT

Nanoparticle-based delivery of self-amplifying RNA

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Several RNA viruses possess a genomic RNA replicon, providing a property of RNA self-amplification [1]. In this context, self-amplifying RNA (saRNA) viruses with a single-stranded RNA of positive polarity comprise of alphaviruses and flaviviruses, while measles viruses and rhabdoviruses carrying a negative-strand RNA genome have been engineered as expression vectors. Several studies in animal models have demonstrated that saRNA viral vectors expressing foreign antigens elicit strong immune responses and can further provide protection of immunized animals against challenges with lethal doses of infectious agents and tumor cells [2]. In addition, saRNA viral vectors have been subjected to several clinical trials targeting both infectious diseases and cancer. For example, a Phase III clinical trial in Guinea and Sierra Leone, provided substantial protection against Ebola virus (EBOV) after a single intramuscular injection with a vesicular stomatitis virus-based vector expressing the EBOV glycoprotein in vaccinated individuals [3]. Related to cancer therapy, measles virus vectors expressing the carcinoma embryonic antigen have been subjected to peritoneal administration in patients with advanced ovarian cancer in a Phase I trial, resulting in no dose-limiting toxicity and stable disease [4]. In another Phase I trial, patients with relapsed refractory myeloma were subjected to intravenous administration of 1×10^{11} $TCID_{50}$ of an oncolytic measles virus vector expressing the human sodium iodide symporter [5]. A complete response was observed in one patient, which persisted for 9 months. Thereafter, an isolated relapse occurred in the skull, which when treated with irradiation allowed the patient to remain disease-free for an additional 19 months.

Despite the excitement of therapeutic applications of saRNAs, issues related to RNA stability and delivery have been of concern. As the focus here is on delivery, it is appropriate to only briefly mention that RNA stability can be improved by engineering of the RNA molecule itself [6]. For instance, engineering of anti-reverse 5′ 7-methylguanosine triphosphate (m7G) Cap analogs (ARCAs) provides more than double RNA transcription efficiency in comparison to conventional cap analogs [7]. Moreover, engineering of the poly(A) tail at the 3′ end of mRNAs has enhanced the stability of RNA [8]. Also, the 5′ and 3′ end untranslated regions have proven important for posttranscriptional regulation of gene expression and might be a target for improvement of mRNA optimization [9]. Finally, chemical modifications of nucleosides such as introducing pseudo-uridine into in vitro transcribed mRNA have been proven to enhance the therapeutic properties of RNA by improving stability and translation [10].

A major factor in achieving success of saRNA-based therapy relates to delivery. Numerous studies in animal models have confirmed efficient delivery and therapeutic activity of saRNAs by replication-deficient and oncolytic viral particles in the fields of infectious diseases and oncology [1]. Although many studies have confirmed that safe application of viral vectors other approaches for RNA delivery including RNA encapsulation technologies have been considered [11]. In this context, cationic liposomes-based nanoparticles have provided protection of mRNA against nuclease degradation and improved cellular uptake [12]. Moreover, efficient in vitro and in vivo delivery of mRNA was established for fully degradable lipid-enveloped pH-responsive polymer nanoparticles [13]. In this context, ovalbumin (OVA) mRNA encapsulated in 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) liposomes injected into mouse ear pinnae, resulted in protection against subcutaneous challenges with EG7-OVA tumor cells [14].

In the context of saRNAs, it has been demonstrated that lipid nanoparticle-based delivery increased immunogenicity compared to unformulated RNA [15]. The Venezuelan equine encephalitis (VEE) virus RNA replicon expressing the Respiratory Syncytial Virus Fusion Glycoprotein elicited broad potent and protective responses in immunized mice. In another study, cationic lipid formulations of

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Classic Swine Fever Virus saRNA replicons encoding influenza A virus nucleoprotein genes were able to target dendritic cells (DCs), critical for enhanced immune defense development [16].

Taking into account the description of therapeutic applications of saRNAs and the evident difficulties related to efficient delivery, the recent timely publication entitled "Inside out: optimization of lipid nanoparticle formulations of exterior complexation and in vivo delivery of saRNA" by Blakney et al. in Gene Therapy provides an interesting alternative approach for lipid nanoparticle formulation [17]. The ingenuity of the approach relates to the formulations with saRNA either on the interior or exterior of particles. Application of different lipids such as ionizable C12–200 and cationic dimethyldioctadecylammonium (DDA) and DOTAP did generate particles in the range of 100–200 nm with a rounded morphology. All lipid nanoparticles (LNPs) with encapsulated saRNAs had a positive surface, ranging from 8 to 30 mV, except for C12-200 LNPs with exterior saRNA, which indicated that the exterior lipid was accessible and not saturated by saRNA. Evaluation of transfection efficiency of a luciferase reporter gene in HEK293 cells revealed that saRNAs complexed to the exterior of C12-200 LNPs was two magnitudes lower than when saRNAs were inside LNPs. In contrast, saRNAs complexed to the exterior of cationic lipids resulted in tenfold higher transfection efficiency compared to interior saRNAs. It was also demonstrated that addition of 50% FCS to the transfection protocol to mimic in vivo conditions of high protein concentration had no effect on transfection efficiency. In addition, LNPs were shown to condense and protect saRNAs from RNase degradation. The LNP formulations were also evaluated for in vivo delivery, which indicated that the composition of cationic lipids played an important role as DDA LNPs showed significantly higher efficiency than DOTAP LNPs. The immunogenicity of cationic LNPs with exterior saRNA carrying the HIV-1 Env gp140 was evaluated in mice, showing specific antibody responses comparable to ionizable LNPs with interior saRNAs. Interestingly, cationic LNP formulations with saRNAs on the surface elicited maximal immune responses after a single immunization and did not require any boosting. Immunization with DDA exterior LNPs exhibited superior antibody titers, which might be contributed to the adjuvant properties of DDA LNPs as previously described for protein vaccines [18]. Another point to address is the relatively short vaccination schedule applied in the study, suggesting that prolongation of the intervals between immunizations could allow for optimization of protein expression and thereby enhance immune responses.

The obvious question which this study raises, relates to what the potential benefits of complexing saRNAs to the surface of LNPs are associated with. It is well documented that LNP production at laboratory scale has encountered batch-to-batch variability related to encapsulation efficiency, size, charge, and RNase contamination [19]. Therefore, the advantage of formulation of LNPs with exterior saRNAs relates to the possibility to perform comprehensive quality control on batches of LNPs prior to the incorporation of saRNA. Perhaps the main advantage relates to the flexibility of engineering LNPs with different RNA constructs, which substantially facilitates and accelerates novel formulations for targeting epidemic outbreaks. It will allow the advance preparation of reproducible LNP batches excluding saRNAs. A similar two-vialed strategy was recently introduced, where a highly stable nanostructured lipid carrier (NLC) was manufactured and stockpiled separately from the target RNA [20]. In this context, VEE saRNA encoding Zika virus antigens was added to the pre-manufactured NLCs, which after immunization provided complete protection of mice challenged with lethal doses of Zika virus.

Overall, application of saRNAs presents an attractive alternative for gene therapy and vaccine development. Already for some time, saRNAs have been administered as naked RNA replicons, recombinant viral particles and layered DNA/RNA replicon vectors providing good safety profiles and protection against challenges with lethal doses of infectious agents and tumor cells in various animal models. Moreover, therapeutic efficacy has been achieved in a limited number of clinical trials. However, to optimize and target saRNA delivery, novel RNA-polymer and RNA-liposome complexes have been formulated. In this context, the inside out LNP formulations have further increased the potency of saRNAs as attractive prophylactic and therapeutic agents for next generation medicines.

Compliance with ethical standards

Conflict of interest The author declares no conflict of interest.

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