

ASSESSMENT OF THE IMMUNOGENICITY OF CLASSIC AND SELF-AMPLIFYING RNA-BASED ANTIVIRAL THERAPEUTICS FOR INFLUENZA

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The high variability of the influenza virus and the limited flexibility of inactivated vaccines result in a need to develop new, more effective preventive therapeutics. The RNA platform is among the most promising ones for developing new generation vaccines against respiratory infections. The study aimed to compare the immunogenicity of two experimental therapeutics based on classic mRNA and self-amplifying RNA (saRNA) encoding the hemagglutinin (HA) of the A(H1N1)pdm09 influenza virus. BALB/c mice were immunized twice with the RNA-based therapeutics at a dose of 2 µg at a 21-day interval. Humoral immune responses were assessed using the hemagglutination inhibition (HAI) assay. After the second immunization, the geometric mean titers of antibodies against the homologous strain A/Wisconsin/588/2019 were 1 : 2281 (CI: 1 : 1319 – 1 : 3943) for the mRNA vaccine and 1 : 640 (CI: 1 : 404 – 1 : 1014) for the saRNA vaccine. Titers against the antigenically distant strain A/Victoria/4897/2022 in the mRNA group and saRNA group reached 1 : 1810 (CI: 1 : 844 – 1 : 3882) and 1 : 452 (CI: 1 : 246 – 1 : 832), respectively. These antibody titer values were considerably higher than those in the group that received the Ultrix Quadri inactivated split vaccine, which did not exceed 1 : 56 (CI: 1 : 26 – 1 : 121). Despite the higher antibody titers in the HAI assay after immunization with the classic mRNA-based vaccine compared with the saRNA-based vaccine, the differences were not statistically significant. Thus, the mRNA- and saRNA-based vaccines induce a pronounced humoral immune response, confirming that these platforms are promising for the development of new generation vaccines.

Keywords: mRNA vaccines, self-amplifying RNA, influenza virus, immunogenicity, hemagglutinin, lipid nanoparticles, cross-reactivity, hemagglutination inhibition

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ОЦЕНКА ИММУНОГЕННОСТИ ПРЕПАРАТОВ ПРОТИВ ВИРУСА ГРИППА НА ОСНОВЕ КЛАССИЧЕСКИХ И САМОАМПЛИФИЦИРУЮЩИХСЯ РНК

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Высокая изменчивость вируса гриппа и ограниченная гибкость инактивированных вакцин обуславливают необходимость в создании новых более эффективных профилактических препаратов. РНК-платформа — одна из наиболее перспективных для создания вакцин нового поколения против респираторных инфекций. Целью работы было сравнить иммуногенность двух экспериментальных препаратов на основе классической мРНК и самоамплифицирующейся РНК (саРНК), кодирующих гемагглютинин (HA) вируса гриппа A(H1N1)pdm09. Мышей линии BALB/c дважды иммунизировали РНК-препаратами в дозе 2 мкг с интервалом в 21 день. Уровень гуморального иммунного ответа оценивали в реакции торможения гемагглютинации. После второй иммунизации среднегеометрические титры антител против гомологичного штамма A/Wisconsin/588/2019 составили 1 : 2281 (ДИ: 1 : 1319 – 1 : 3943) для мРНК и 1 : 640 (ДИ: 1 : 404 – 1 : 1014) для саРНК. Против антигенно-дистантного штамма A/Victoria/4897/2022 титры в группах мРНК и саРНК достигли 1 : 1810 (ДИ: 1 : 844 – 1 : 3882) и 1 : 452 (ДИ: 1 : 246 – 1 : 832) соответственно. Эти показатели титров антител были значительно выше, чем в группе инактивированной сплит-вакцины «Ультрикс Квадри», которые не превышали 1 : 56 (ДИ: 1 : 26 – 1 : 121). Несмотря на более высокие титры антител в РТГА после иммунизации вакциной на основе классической мРНК по сравнению с саРНК, различия в титрах антител не достигали статистической значимости. Таким образом, вакцины на основе мРНК и саРНК приводят к формированию выраженного гуморального ответа, что подтверждает их перспективность для создания вакцин нового поколения.

Ключевые слова: мРНК-вакцины, самоамплифицирующаяся РНК, вирус гриппа, иммуногенность, гемагглютинин, липидные наночастицы, перекрестная реактивность, реакция торможения гемагглютинации

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Influenza viruses causing seasonal illnesses belong to the *Orthomyxoviridae* family, which is divided into four genera (A, B, C, and D). Influenza A virus causes the most severe infections and is responsible for 290,000–650,000 deaths annually, primarily among children, pregnant women, and the elderly [1]. The high variability of the virus is determined by various mechanisms, including antigenic drift (mutations in the genes encoding viral proteins) and shift (reassortment of genome segments), which results in the need for the annual update of the vaccine antigenic composition [2–4]. Thus, these specific characteristics of the influenza virus necessitate the periodic composition update of vaccines against its constantly changing antigenic determinants.

Prophylactic vaccination remains the most effective tool for controlling viral infections, as it reduces disease incidence and the severity of clinical manifestations [5]. Today, inactivated vaccines such as Ultrix Quadri, Sovigripp, Flu-M, Grippol Plus, Grippol, and Quadrivalent are used to prevent influenza in Russia [6]. Despite a good safety profile, inactivated vaccines have a number of limitations. These include weak cross-reactivity of the induced immune response, the need for annual updates to the strain composition, partly due to the waning of immunity over the course of a year, reduced efficacy in certain at-risk groups, and a long production lead time that hampers fast adaptation to circulating virus variants [1]. Moreover, viral antigens may be altered during the manufacturing of inactivated vaccines, which may reduce the epidemiological efficacy of vaccination campaigns [7, 8]. Revaccination is usually necessary to maintain long-term protection [1]. It has been shown that the efficacy of inactivated vaccines ranges from 10 to 60% due to the lengthy vaccine development process, as well as alterations in the antigenic determinants of the influenza virus [9].

In contrast to inactivated vaccines, RNA vaccines encode the antigen's native structure, possess intrinsic adjuvant properties, and allow rapid sequence adaptation in response to viral variability [10, 11]. Classic mRNA molecules and self-amplifying RNA (saRNA) can be used as a basis for RNA vaccines. saRNA comprises both the sequence of the target antigen and sequences of non-structural alphavirus genes that ensure RNA replication within the cell [11, 12]. saRNA-based therapeutics provide more prolonged target protein biosynthesis, leading to higher immunogenicity or enabling a reduction in dose in the context of vaccines. However, the use of saRNA-based therapeutics is still limited due to potential side effects associated with increased reactogenicity. The study aimed to conduct a comparative analysis of the immunogenicity of experimental therapeutics based on classic mRNA and self-amplifying RNA encoding the hemagglutinin of the A(H1N1)pdm09 influenza virus in BALB/c mice. The licensed Ultrix Quadri inactivated vaccine was used as a positive control to validate the experimental system and ensure correct interpretation of the data. Dynamic changes in the humoral immune response (antibody titers in the HAI assay) were assessed after two-dose immunization at a 21-day interval. The study focused on determining the potential of the saRNA platform for influenza vaccine development.

METHODS

Cloning and *in vitro* transcription

The plasmid encoding the self-amplifying RNA that contained the Venezuelan equine encephalitis (VEE) virus replicon with the *GFP* gene (T7-VEE-GFP) was obtained from Addgene (plasmid

No. 58977). The source plasmid containing the EMCV IRES sequences and the puromycin resistance gene was modified by deleting these elements. The S773P mutation was introduced into the sequence of the nsP2 non-structural protein to reduce reactogenicity. Furthermore, the eGFP sequence under the control of the subgenomic promoter was replaced with the hemagglutinin sequence of the A/Wisconsin/588/2019 (H1N1) influenza virus. To construct a classic messenger RNA, the HA sequence was flanked by the 5' and 3' untranslated regions (UTRs) of human alpha-globin, as well as a poly(A) tail of 110 nucleotides.

In vitro transcription of saRNA and mRNA was performed in a reaction mixture containing 5 µg of the linearized plasmid, reagents from the RNA-20 Kit (Biolabmix, Russia), 2.4 mM of the synthetic analog 3'-O-Me-m7G(5')ppp(5')G (ARCA cap analog) (Biolabmix, Russia) for saRNA, and 2.4 mM of m7G(5')ppp(5')AmG (CapAG; Biolabmix) for mRNA, 1 µg/µL of RiboCare ribonuclease inhibitor (Evrogen, Russia), and 0.002 U/mL of inorganic pyrophosphatase (New England Biolabs, USA) according to the manufacturer's protocol. The *in vitro* transcription of saRNA was performed using a standard nucleotide mix containing 3 mM each of ATP, UTP, CTP and 0.6 mM GTP (Biolabmix, Russia); mRNA synthesis was performed using 3 mM each of ATP, CTP, GTP and N1-methylpseudouridine (Biolabmix, Russia). The reaction was carried out for 1 h at 37 °C. To remove the DNA template, the samples were treated with thermolabile DNase (Thermolabile DNase; Biolabmix, Russia). Subsequently, RNA was purified using magnetic beads for RNA isolation (RNA beads; Vazyme, China). The length and purity of the synthesized RNA molecules were assessed using a MultiNA microchip electrophoresis system (Shimadzu, Japan).

Formulation of RNA into lipid nanoparticles

An automated NP System for liposome generation (microchip mixer, Dolomite Microfluidics, UK) was used for mRNA and saRNA encapsulation into lipid nanoparticles (LNPs). The formulation method was similar to that reported for other mRNA platforms [13]. The lipid composition consisted of the following components: cholesterol (Merck, USA), the ionizable cationic lipids (ICLs) SM-102 and ALC-0315 (Sinopeg, China), DSPC auxiliary lipid (Avanti, USA), DMG-PEG2000 pegylated lipid (Avanti, USA). The optimal molar ratio of the components was 42.70:23.15:23.15:9.40:1.60 (cholesterol : SM-102 : ALC-0315 : DSPC : DMG-PEG2000).

After formulation, the particles suspensions were purified by dialysis using 1 mL Float-A-Lyzer G2 membranes with a 10kDa molecular weight cut-off (MWCO); the membrane was made of the cellulose ester (Repligen, USA). The membranes were prepared according to the manufacturer's protocol. Dialysis was performed overnight (12 h) in phosphate-buffered saline (PBS, pH 7.4) at +4 °C with vigorous stirring. The buffer was replaced once. Cryoprotection was achieved by incubating the samples in 20% sucrose in PBS for 2 h at +4 °C; subsequently, aliquots for analysis and functional testing were frozen at –80 °C.

Analytical characterization of RNA therapeutics

The size and polydispersity index (PDI) of the lipid nanoparticles (LNPs) were determined using a Zetasizer Ultra analyzer (Malvern Panalytical). LNP samples were pre-diluted 100-fold with phosphate-buffered saline (PBS) and kept at room temperature for thermal equilibration. Measurements were performed in plastic cuvettes; three replicates were recorded for each sample (up to 100 scans per replicate) to achieve stable readings.

The encapsulation efficiency (EE, %) was determined according to the published protocol [14]. To assess encapsulation, LNPs were lysed in TE buffer (pH 7.5) containing 1% Triton X-100. Staining with the SYBR Green intercalating fluorescent dye (Evrogen, Russia) was performed for both intact and lysed particles, corresponding to before and after lipid membrane destabilization. The RNA quantity was determined from a calibration curve generated using the RNA standard in TE buffer (with and without Triton X-100). The final concentration of encapsulated RNA was calculated as the difference between the signals obtained for lysed and native samples.

The detailed characteristics of the mRNA-LNP therapeutics used in the experiment are provided in the Results.

Immunization

To assess immunogenicity, four groups of BALB/c mice were formed (females aged 7–8 weeks, six animals per group; Fig. 1A). Mice were randomized into groups and immunized intramuscularly into the thigh muscle on days 0 and 21 with 2 µg of saRNA, 2 µg of mRNA, 3 µg (0.2 human dose) of the Ultrix Quadri vaccine (strain composition 2025–2026 for the Northern Hemisphere: A/Victoria/4897/2022(H1N1)pdm09; A/Croatia/10136RV/2023(H3N2); B/Austria/1359417/2021; B/Phuket/3073/2013), or 100 µL of placebo (PBS with 20% sucrose). Serum antibody titers were determined by hemagglutination inhibition (HAI) assay on day 20 after the first immunization and on day 21 after the second immunization (day 42 after the first immunization) against the hemagglutinin of the antigenic variants of the A(H1N1)pdm09 influenza virus.

The dose of the Ultrix Quadri inactivated vaccine for mice (3 µg of hemagglutinin per strain, corresponding to ~0.2 human dose) was selected based on allometric scaling by body surface area using Km factors [15]: Km (human) = 37, Km (mouse) = 3. The estimated equivalent dose was ~1.2 µg. However, a conservative dose of 3 µg was used to ensure reliable detection of the immune response and comparability with the dose of the experimental RNA therapeutics (2 µg). Since the experimental RNA therapeutics encode only one antigen (HA of A/Wisconsin/588/2019), immunogenicity was compared with that of Ultrix Quadri based on the equivalent dose of hemagglutinin of the corresponding H1N1 strain, ensuring correct interpretation of the data.

Hemagglutination inhibition (HAI) assay

In animal experiments, immunogenicity was assessed using the hemagglutination inhibition (HAI) assay according to a protocol based on the World Health Organization (WHO) protocol [16]. For this purpose, mice sera were treated with the receptor-destroying enzyme (RDE) obtained from Vibrio cholerae, which was purchased from Denka Seiken Co., Ltd. (Tokyo, Japan). Two-fold dilutions of the treated sera were prepared in 96-well V-bottom plates. Subsequently, the viral antigen at a working dose of 8 agglutination units (AU) was added, and the plate was incubated for 30 min at room temperature. A 0.5–1% suspension of human group O red blood cells (ABO system) was then added, and the plate was incubated for another 60 min at room temperature. When antibodies capable of cross-reacting with the virus were present in the serum sample, they bound to the virus and inhibited its ability to agglutinate red blood cells. After incubation, the antibody titer was determined as the highest serum dilution at which inhibition of hemagglutination was still observed.

The A/Victoria/4897/2022 influenza virus antigen for the HAI assay was purchased from Diagnostic Drug Manufacturing Plant LLC (Saint Petersburg, Russia). To accumulate sufficient antigenic material, the A/Wisconsin/588/2019 strain was propagated in MDCK cell culture according to a standard method [16].

Statistical analysis

Statistical analysis was performed using the Kruskal-Wallis one-way analysis of variance by ranks test and Dunn's test for pairwise comparisons (post hoc). The differences between experimental groups were considered significant at $p < 0.05$. Data were analyzed and visualized using GraphPad Prism 10.4.1 (GraphPad Software, USA).

RESULTS

In the first phase, we assessed the quality of the obtained mRNA- and saRNA-based vaccines encapsulated in lipid nanoparticles (Fig. 1). Both therapeutics had an RNA concentration of 20 ng/µL. The therapeutics showed high encapsulation efficiency: 99.9% for classic mRNA and 94.0% for saRNA (Fig. 1A). The average hydrodynamic diameters were comparable: ~99.2 nm for the mRNA-based therapeutic and ~88.2 nm for the saRNA-based therapeutic, which are within the optimal size range for cellular uptake via endocytosis. The integrity and size of the nucleic acid were verified by capillary gel electrophoresis after destruction of the lipid nanoparticles. Clear, discrete bands without signs of degradation indicated that the obtained therapeutics were of high quality. The length of the classic mRNA was ~2000 nucleotides, and that of the saRNA was ~9500 nucleotides, owing to the presence of non-structural alphavirus genes in its sequence (Fig. 1B). Analysis of the particle size distribution by dynamic light scattering confirmed that both therapeutics were homogeneous. The particle size ranged from 88 to 100 nm, and the polydispersity index (PDI) was < 0.11 (Fig. 1C, D). Thus, the mRNA- and saRNA-based therapeutics showed high-quality indicators and comparable physical and chemical parameters, which allowed us to use them for further immunogenicity assessment.

Twenty days after the first immunization, detectable titers of hemagglutination-inhibiting antibodies ($p < 0.01$) against the A/Wisconsin/588/2019 and A/Victoria/4897/2022 influenza virus antigens were observed in the groups of mice that received experimental RNA therapeutics (saRNA and mRNA), compared with the negative control group (PBS) (Fig. 2B). In the saRNA group, the geometric mean titers of antibodies against the A/Wisconsin/588/2019 strain were 1 : 180 (CI (95% confidence interval): 1 : 133 – 1 : 242), and those against the A/Victoria/4897/2022 strain were 1:67 (CI: 1 : 44 – 1 : 92). In the classic mRNA group, higher values for antibodies were observed: 1 : 285 (CI: 1 : 165 – 1 : 493) and 1 : 101 (CI: 1 : 48 – 1 : 214) against the same strains, respectively. However, the differences between the groups were not statistically significant. In the group of animals immunized with the Ultrix Quadri commercial inactivated split vaccine (0.2 human dose), the HAI antibody titer against A/Wisconsin/588/2019 was 1 : 71 (CI: 1 : 53 – 1 : 96), whereas antibody titers against the A/Victoria/4897/2022 strain were minimal (below 1 : 37 (CI: 1 : 17 – 1 : 73)). Thus, although the Ultrix Quadri vaccine comprised antigens of the A/Victoria/4897/2022 strain, it induced a weaker immune response at the selected dose.

Twenty days after the second immunization (day 41 of the experiment; Fig. 2C), a considerable increase in the

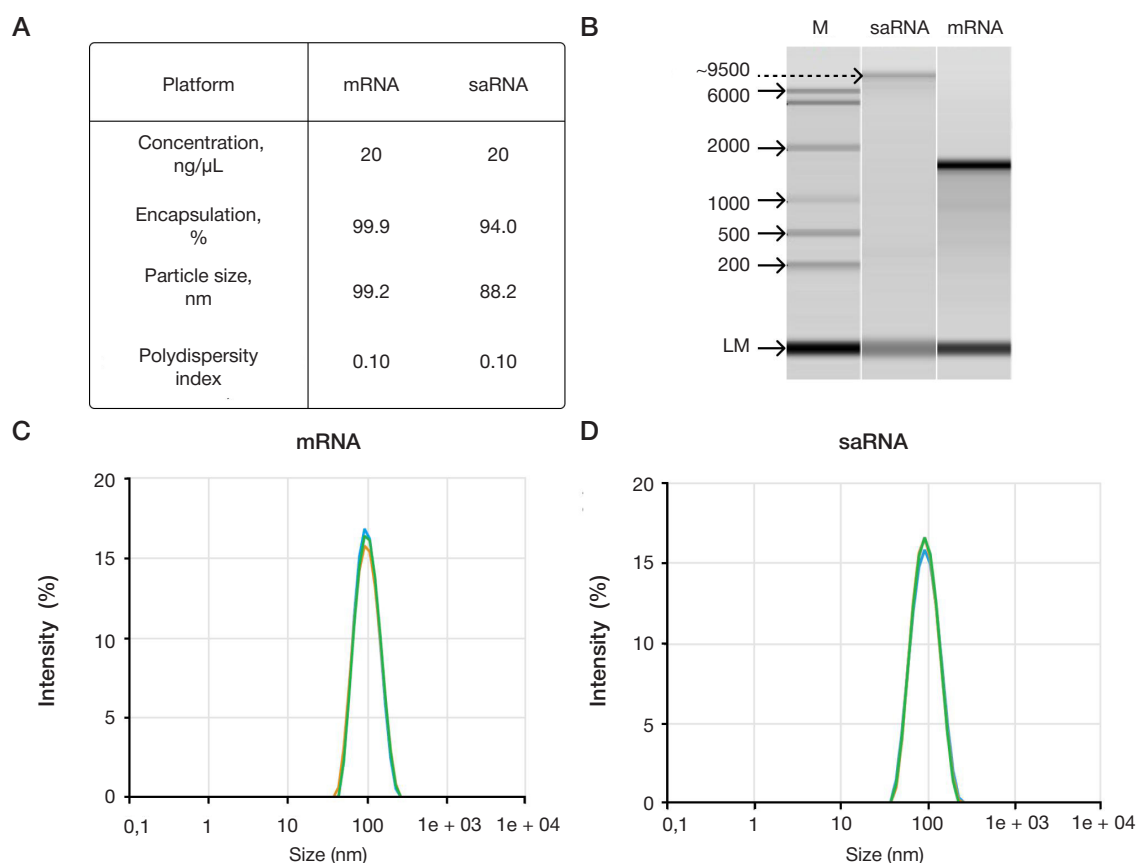


Fig. 1. Physicochemical parameters of RNA-LNPs. **A.** Analytical characteristics of lipid nanoparticles. **B.** Capillary electrophoresis of saRNA and mRNA after particle destruction. The expected saRNA length is indicated by a dotted arrow. **C, D.** Particle size distribution by dynamic light scattering: mRNA and saRNA, respectively

titers of hemagglutination-inhibiting antibodies against both the A/Wisconsin/588/2019 and A/Victoria/4897/2022 strains, compared with the first immunization (Fig. 2B), was observed in the groups that received the experimental RNA therapeutics. The most pronounced increase in antibody titers was observed in the group of mice immunized with the classic mRNA vaccine. In the mRNA group, the antibody titer against A/Wisconsin/588/2019 was 1 : 2281 (CI: 1 : 1319 – 1 : 3943), which was significantly higher ($p < 0.0001$) than those in the negative control group (PBS) and the group of mice that received the control vaccine (1 : 17 (CI: 1 : 8 – 1 : 42)). The mRNA group also showed high antibody titers against the A/Victoria/4897/2022 strain (1 : 1810 (CI: 1 : 844 – 1 : 3882)), which were significantly higher than those in the negative control group and the Ultrix Quadri group (1 : 56 (CI: 1 : 26 – 1 : 121); $p < 0.05$). In the saRNA group, antibody titers also increased after the second immunization, but the increase was less pronounced than in the mRNA group. In the saRNA group, the antibody titer against the A/Wisconsin/588/2019 strain was 1 : 640 (CI: 1 : 404 – 1 : 1014) and that against the A/Victoria/4897/2022 was 1 : 452 (CI: 1 : 246 – 1 : 832); both were significantly higher than those in the control group ($p < 0.05$). The differences between the saRNA and mRNA groups were not statistically significant. After the second immunization, the Ultrix Quadri vaccine induced low HAI antibody titers, which were considerably lower than those in both RNA vaccine groups. Overall, these data indicate that mRNA- and saRNA-based vaccines elicit a robust humoral immune response.

DISCUSSION

The study involved a comparison of the immunogenicity of experimental vaccines based on the classic mRNA and

self-amplifying RNA encoding the hemagglutinin (HA) of the A/Wisconsin/588/2019 (H1N1)pdm09 influenza virus in a preclinical BALB/c mouse model. After the second immunization, a considerable increase in antibody titers was observed in both RNA vaccine groups; these titers were significantly higher compared with those observed with the Ultrix Quadri vaccine. The latter induced only a moderate increase or no significant immune response to the A/Wisconsin/588/2019 and A/Victoria/4897/2022 strains. The low immunogenicity of the inactivated vaccine observed in these experiments may result from both the low vaccine dose used (3 μ g of each of four antigens) and the inherent properties of the platform (fixed antigen dose, lack of endogenous expression, and lack of adjuvant). As for RNA-based therapeutics, according to the literature, these ensure prolonged biosynthesis of viral antigens and may also provide an adjuvant effect through the activation of innate immune receptors (TLR3, TLR7/8, RIG-I) [11].

The cross-reactivity analysis showed that RNA-based therapeutics induce high antibody titers against both the homologous A/Wisconsin/588/2019 strain and the antigenically distant A/Victoria/4897/2022 variant. The ability of both RNA therapeutics to induce a pronounced immune response against the antigenically distant A/Victoria/4897/2022 strain confirms the potential of the RNA platform for inducing cross-reactive immune responses [17]. These findings are consistent with published data demonstrating that RNA vaccines can elicit antibodies that recognize antigenically drifted strains [18–20]. Thus, the generation of multivalent constructs encoding hemagglutinins of several influenza subtypes may contribute to the development of a universal vaccine that provides broad cross-protection [21]. These results confirm the promise of both RNA-based technologies for influenza prevention.

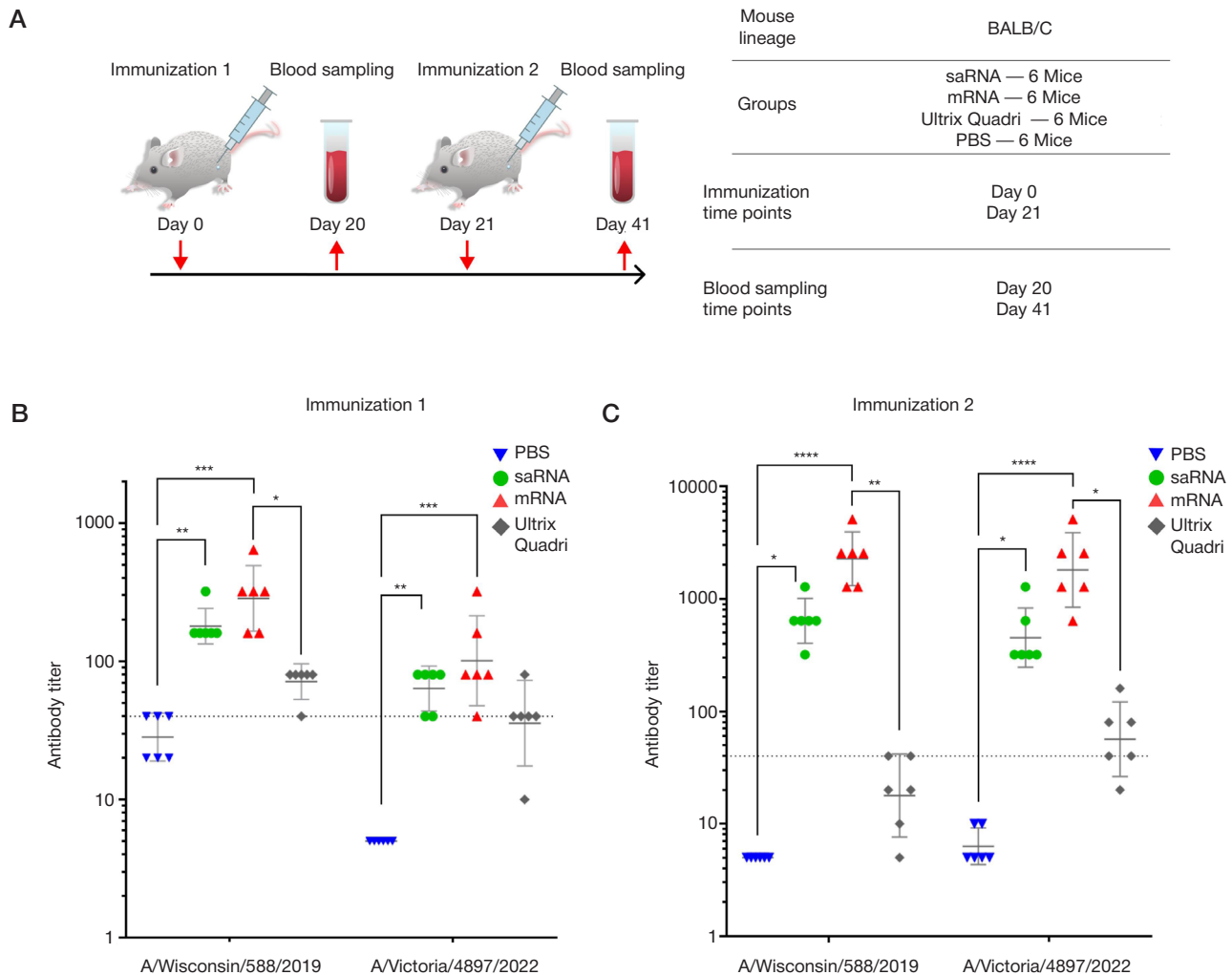


Fig. 2. Immunization schedule and HAI assay results. **A.** Experimental design. **B.** Graph of the geometric mean antibody titers in the groups of mice immunized with PBS, mRNA, saRNA, and Ultrix Quadri 20 days after the first immunization. **C.** Graph of the geometric mean antibody titers in the groups of mice immunized with PBS, mRNA, saRNA, and Ultrix Quadri 21 days after the second immunization (* — $p < 0.05$; ** — $p < 0.01$; *** — $p < 0.001$; **** — $p < 0.0001$; Dunn's test).

The HAI antibody titers for the classic mRNA vaccine obtained in our study (1 : 2281 (CI: 1 : 1319 – 1 : 3943) against the homologous strain after the second immunization) are within the range of values reported in other studies [19, 20]. It has been shown that a single-dose immunization with an RNA-based influenza therapeutic induced titers $>1 : 40$, while after the second immunization the values were in the range of 1 : 1000 to 1 : 5000, depending on the strain and dose [22]. In our previous study [23], geometric mean HAI antibody titers after immunization with two doses of a trivalent mRNA vaccine were also 1 : 1000 and ~86-fold higher than those induced by the inactivated vaccine. As for the saRNA platform, our data (1 : 640 (CI: 1 : 404 – 1 : 1014) at a dose of 2 μg) are consistent with data from another study showing that self-amplifying RNA-based vaccines induce high HAI titers after the first immunization: approximately $1 : 104 \pm 53.67$ at a dose of 1.25 μg [24]. Despite the lack of significant differences in HAI assay titers in the mRNA and saRNA groups, the absolute values and the range were higher in the mRNA group. This may be attributed to the specific translation pattern of saRNA. Indeed, *in vivo* bioluminescence imaging of reporter proteins has shown that lower levels of target protein biosynthesis are observed in the first days after the saRNA administration, reaching a maximum on days 5–7 and then gradually declining [25–27]. In contrast, for mRNA-based therapeutics, maximum reporter protein expression is reached 24 h after administration and

then decreases considerably, owing to the short half-life of the mRNA [25–27].

The lack of assessment of cellular immune responses is an important limitation of this study. In this study, we focused on assessing humoral immunity, particularly on evaluating hemagglutination-inhibiting antibodies. HAI antibody titers correlate with protection against influenza and are used by regulatory authorities to assess vaccine efficacy [28]. Furthermore, at the same mass dose (2 μg), the molar amount of saRNA (~9.5 kb) was approximately five times lower than that of mRNA (~2 kb). To detect possible dose-dependent side effects, it will be necessary to compare equivalent molar doses of the therapeutics. The dose selection was constrained by the reactogenicity of the saRNA platform (due to innate immune activation by replication intermediates) and by the compensatory effect of self-amplification, which ensures high antigen expression even from a lower initial number of molecules.

Despite the more modest immunogenicity of the candidate saRNA-based vaccine, its use offers greater potential for dose reduction and may also contribute to a more prolonged maintenance of antibody titers compared with mRNA, owing to intracellular replication, potentially reducing the cost of the medicinal product and the side effects associated with higher lipid nanoparticle doses. The prospects for further development lie in optimizing the self-amplifying RNA sequence. In particular, introducing mutations into the sequences of the replicon's

non-structural proteins and optimizing regulatory elements can significantly improve the immunogenicity of the saRNA vaccines [11].

CONCLUSIONS

The data obtained suggest that the candidate RNA therapeutics based on the classic mRNA and saRNA, encapsulated into

lipid nanoparticles, induce a pronounced humoral immune response to the homologous A/Wisconsin/588/2019 strain and the antigenically distant A/Victoria/4897/2022 strain, highlighting their potential for the development of new-generation vaccines against seasonal and pandemic influenza. Our findings show that the candidate saRNA-based influenza vaccine is not superior to the classic mRNA-based vaccine. Thus, further optimization of its sequence, dose, and route of administration is necessary.

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