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The SARS-CoV-2 rS1-E-PLGA nanovaccine and evaluation of its immune effect in BALB/c mice

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Abstract. – OBJECTIVE: Vaccination is an important method for preventing COVID-19 infection. However, certain vaccines do not meet the current needs. To improve the vaccine effect, discard ineffective antigens, and focus on high-quality antigenic clusters, S1-E bivalent antigens were designed.

MATERIALS AND METHODS: Vaccine delivery is performed using poly (lactic-co-glycolic acid) (PLGA). Here, the recombinant S1-E (rS1-E) was covered on PLGA and injected intramuscularly into mice. In total, 48 BALB/c mice were randomly divided into six groups with 8 mice in each group. The mice received intramuscular injections. Prior to vaccination, the hydrophobicity of the rS1-E and the antigenic site of the E protein were both analysed. The morphology, zeta potential, and particle size distribution of rS1-E-PLGA were examined. Anti-S1 and anti-E antibodies were detected in mouse serum by ELISA. Neutralising an-tibodies were detected by co-incubating the pseudovirus with the obtained serum. IL-2 and TNF-α levels were also measured.

RESULTS: The designed recombinant S1-E protein was successfully coated on PLGA nanoparticles. rS1-E-PLGA nanovaccine has suitable size, shape, good stability, sustained release and other characteristics. Importantly, mice were stimulated with rS1-E-PLGA nanovaccines to produce high-titre antibodies and a good cellular immune response.

CONCLUSIONS: Our results indicate that rS1-E-PLGA nanovaccine may provide a good protective effect, and the vaccine should be further investigated in human clinical trials for use in vaccination or as a booster.

Key Words:
COVID-19, Enevolpe protein, Spike protein, Nanovaccine, PLGA.

Introduction

COVID-19, a disease caused by SARS-CoV-2 infection, was first identified in 2019 and quickly spread throughout the world[1]. There are a few drugs that specifically treat COVID-19. Vaccines have become the first choice for the prevention and treatment of COVID-19 in the absence of specific therapeutic medicines[2].

The S1 subunit of SARS-CoV-2 is an important target for vaccine development[3]. S1 exhibits a receptor-binding function and contains a signal peptide at the N-terminus. And it also possesses an N-terminal domain and a receptor-binding domain (RBD)[4]. As the outermost part of SARS-CoV-2, the S1 subunit is more easily exposed *in vivo*. It is a target for vaccine research directed toward not only more easily activating the immune system to produce antibodies but also to causing these antibodies to be more specific and effective. However, S1 of SARS-CoV-2 is prone to mutation. Most reported variants possess mutations in S1. The presence of anti-SARS-CoV-2 S1 antibodies alone may not be effective in preventing SARS-CoV-2 infection. Other SARS-CoV-2 protein structures may also need to be discovered for use in developing vaccines. The base sequence of the envelope protein is highly conserved[5]. The E protein is a SARS-CoV-2 membrane protein that is involved in virulence, and this protein forms holes in the host membrane and can cause inflammation[6]. It is predicted that some overlapping CTL, HTL, and B cell epitopes on E protein could be potential general candidates for vaccine development. Sequence alignment of the E protein of four different strains of coronavirus revealed that compared to other structural proteins

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of SARS-CoV-2, the envelope protein is relatively conserved and possesses higher immunogenicity[7]. A more antigenic S1 structure and a more conserved E structure are integrated in the rS1-E fusion protein.

Nanovaccine is a new generation of vaccines that use nanoparticles (NPs) as carriers and/or adjuvants. The body is stimulated by antigens covered in NPs to produce improved cellular and humoral immunity[8]. PLGA is one of the most successfully biodegradable polymers. As an antigen delivery system, it possesses numerous advantages, including (I) biocompatibility and biodegradability, (II) FDA and the European medical agency approval of the drug delivery system, utility for parenteral drug delivery, (III) ease of preparation and good applicability as drug delivery, (IV) the ability to help reduce the degradation of vaccine antigens, (V) the possibility of a sustained-release, and (VI) variability of surface properties with improved interactions[9,10].

To date, there is little information regarding the E antigen and no information regarding the S1-E antigen as a SARS-CoV-2 vaccine candidate. We have developed a vaccine as a candidate or booster for the re-emerging COVID-19 outbreak.

Materials and Methods

Ethics Statement

All animal experiments were complied with the ARRIVE guidelines and were performed in accordance with the UK Animals (Scientific Procedures) Act of 1986 and associated guidelines according to EU Directive 2010/63/EU for animal experiments. All experimental protocols in Hunan Province were approved by the Science and Technology Agency. The approval ID was SYXK (Xiang) 2020-0002.

Materials

PLGA (D,L-lactic acid-co-glycolide 65:35, Mw=40,000-75,000, CAS#26780-50-7) and (PVA Mw=20,000–40,000, CAS#9002-89-5) were purchased from Coolaber (Beijing, China). Complete Freund's adjuvant (Cat#F5881) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Freund's incomplete adjuvant (CAT#B545288, Sangon Biotech, Shanghai, China), HEK-293T/ACE2 cells (CAT#nCov-3), and SARS-CoV-2_del19AA-GFP pseudovirus (CAT#LV-nCov2) were purchased from Packgene, Guangzhou, Guangdong, China. pET-28a(+) recombinant plasmid (clone ID:TG418436B) was obtained from GenScript Biotech, Nanjing, Jiangsu, China. The E protein (CAT#ATEP02466COV) was purchased from Atagenix, Wuhan, Hubei, China. The SARS-CoV-2 (2019-nCoV) Spike S1 Antibody Titre Assay Kit (Mouse, CAT#KIT007) was purchased from Sinobiological, Beijing, China. The Ni^{2+} affinity chromatography column (CAT#30210) was purchased from Qiagen (Germantown, MD, USA).

In total, 48 specific pathogen-free (SPF) female BALB/c mice (age:6-8 weeks, body weight:17-20 g) were purchased from Hunan SJA laboratory animal CO., LTD, China (Qualified Certificate: SCXK [Xiang] 2019-0004).

Construction and Expression of rS1-E Plasmid of SARS-CoV-2

The rS1-E plasmid of SARS-CoV-2 was constructed in our lab. Based on our previous experience, the recombinant protein gene carrying the recombinant plasmid was expressed. Recombinant protein was purified as previously described[11]. Briefly, the recombinant plasmid pET-32a(+) containing rS1-E was transformed into Rosetta (DE3) cells and then cultured in Luria Bertini (LB) medium containing kanamycin (100 μg/mL). Expression was induced with isopropyl-β-D-thiogalactoside (IPTG) at a concentration of 0.5 mM for 6 h at 30ºC. The recombinant protein was expressed as inclusion body protein. It was dissolved in 6M guanidine hydrochloride (pH 8). The protein was purified using Ni^{2+} affinity chromatography. The eluted proteins were dialysed and vacuum dried. The rS1-E was identified using SDS-PAGE and Coomassie Brilliant Blue (CAS#6104-59-2, Solarbio, Beijing, China) staining.

Preparation of rS1-E-PLGA Nanovaccine

PLGA (50 mg) and rS1-E were added to an organic solution of dichloromethane (900 μL) and acetone (100 μL) that formed the first emulsion by ultrasonic emulsification. The first emulsion and PVA aqueous solution (20 g/L) were then phacoemulsified for a second time to form the final emulsion. Finally, the emulsion and water were stirred for 4 h and washed with PBS centrifugation 3 times. The nanoparticle powders were subjected to Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and

assessed to determine drug loading capacity (LC) and embedding ratio (ER).

LC (%) = weight of protein in nanoparticles/ weight of nanoparticles × 100.

ER (%) = weight of protein in the nanoparticles/ weight of the feeding protein × 100%.

The remaining nanovaccine was lyophilised by vacuum drying, and a portion of the nanovaccine was lyophilised for SEM detection, zeta potential detection, and particle size distribution

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In Vitro Release Test

To assess the extraneous release of S1-E-loaded PLGA, 20 mg of rS1-E-PLGA NPs was added to 10 ml of PBS solution to form solution 1. Solution 1 was placed in a slow shock water bath at 37ºC. One millilitre of supernatant was tested for protein quality, and 1 ml of fresh PBS was added to the original container. The supernatant was centrifuged at 13,000 rpm every 12 h to detect the protein concentration. The data were recorded and graphed. The experiment was repeated three times.

Vaccination and Stimulation of Mice

Female BALB/c mice (6-8 weeks old) were randomly divided into six groups for animal experiments. At weeks 0, 3, and 6, the mice were immunised by intramuscular injection of 40 μg of rS1-E-PLGA nanoparticles, 40 μg (endotoxin content 1.92 μg) of recombinant rS1-E, 40 μg of S1-E Freund's adjuvant (endotoxin content 1.92 μg), PBS+PLGA (40 μg), 200 μL of PBS (2% polymyxin B [CAS#1405-20-5] was added to all vaccines), and 40 μg of recombinant S protein. Various vaccines were added to PBS at a volume of 200 μL. Blood samples were collected prior to each immunization, and the serum was separated for antibody detection. Serum samples were stored at -20ºC until use.

Antibody Assays

The serum of the mice was collected at 0, 2, 4, 6, 8, and 10 weeks. Serum was obtained by cutting the tail at 0, 2, 4, 6, and 8 wk. Serum was obtained by eye extraction, and spleen cells were obtained by dissecting the mouse. Anti-S1 antibody was detected using a commercially available mouse enzyme-linked immunoassay (ELISA) kit. According to the operation steps of the kit, E protein was adsorbed on a transparent enzyme-labelled plate and incubated at 37ºC for 1 h. The plate was then washed with PBST 5 times. The plates were sealed overnight and incubated with 1% bovine serum albumin (BSA). The plate was washed with PBST 5 times, and gradient dilution serum was added and incubated at 37ºC for 2 h. The plate was washed with PBST 5 times. HRP-labelled sheep anti-mouse IgG antibody was then added, and the plate was incubated at 37ºC for 1 h. The plate was then washed with PBST 5 times. TMB solution was added and incubated in the dark for 20 min, and then H_2SO_4 was added to terminate colour development. When the OD_{450} of the serum/control was >2.1, it was considered positive.

SARS-CoV-2-S1 Pseudovirus Neutralization Assay

A total of 1.5×10^4 HEK-293T/ACE2 cells/well were segregated into the culture plate and cultured in a constant temperature incubator at 37°C and 5% CO_2 for 6 h. The serum was inactivated in a water bath at 56°C for 30 min, and the sample serum was continuously diluted with 10% DMEM. At each dilution, 60 μl of serum samples were mixed with the same volume of pseudovirus that was diluted to MOI=0.2, and these mixtures were incubated at room temperature for 60 min. A mixture of 100 μl of incubated serum and pseudovirus was added to a 96-well cell culture plate, and the negative control and blank control were set. After 72 h, cells were observed under a fluorescence microscope. EC_{50} neutralisation titres were calculated for each mouse serum sample using the Reed-Muench method[12].

Intracellular Cytokine Staining

Splenocytes from BALB/c mice were prepared by pushing the spleen through a 70-μm cell strainer, and this was followed by red blood cell lysis and several washes. The cells were stimulated for 6 h at 37°C with or without 1 μg/mL of overlapping 15-amino-acid peptides covering the rS1-E and with BD (Franklin Lakes, NJ, USA) GolgiStop™ and BD GolgiPlug™ to block cytokine secretion. Following stimulation in a petri dish, the splenocytes were washed and stained with a mixture of antibodies specific for lineage markers, including FITC hamster anti-mouse CD3e (clone 145-2C11, 1:200 dilution), APC rat anti-mouse CD4 (clone RM4-5, 1:200 dilution), and PE rat anti-mouse CD8a (clone 53-6.7, 1:200 dilution). After one wash with PBS, the cells were fixed and permeabilised with Cytofix/Cytoperm (BD Biosciences), washed with Perm/Wash buffer (BD Biosciences, Franklin Lakes, NJ, USA), and stained with Per-

CP-Cy™5.5 rat anti-mouse IL-2 (clone JES6-5H4, 1:200 dilution) and PerCP-Cy™5.5 rat anti-mouse TNF (clone MP6-XT22, 1:200 dilution). The cells were washed successively with Perm/Wash buffer and PBS and then resuspended in PBS. At least 10,000 events were recorded for each sample. $CD8^+$ and $CD4^+$T cells were gated from single cells (FSC-A vs FSC-H), lymphocytes (FSC-A vs SSC-A), and live $CD3^+$T cells ($CD3^+$vs Near-IR–), successively, and the detection results were defined as the percentage of cytokine-positive cells among $CD8^+$or $CD4^+$T cells (the average value of the PBS group was set to "0").

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.0.2 (La Jolla, CA, USA). $p < 0.05$ was considered statistically significant. Unpaired *t*-test was applied to compare differences between two groups, and ordinary one-way ANOVA was used to compare >2 groups.

Results

The Vector SARS-CoV-2 S1-E was Successfully Constructed, and the rS1-E was Expressed in E. coli

The genome sequence for SARS-CoV-2 was queried in the NCBI database, and the gene sequences for the S1 and E proteins were screened using the UniProt database. The two sequences were connected by a linker to construct the pET-28a(+) recombinant plasmid (Figure 1a). The hydrophobicity of the rS1-E was predicted (Figure 1b), and it was determined that the E protein was hydrophobic and possessed a peak hydrophobicity score of 3.5. The average hydrophobicity of the sequence was -0.106 according to the calculation method based on Kyte and Doolittle (1982)[13]. The epitope of the sequence was predicted using online analysis tools (Figure 1c). The epitope scores for the 38 sequences were satisfactory.

The synthetic gene sequence was transformed into Rosetta (DE3) cells. Rosetta cells were in-

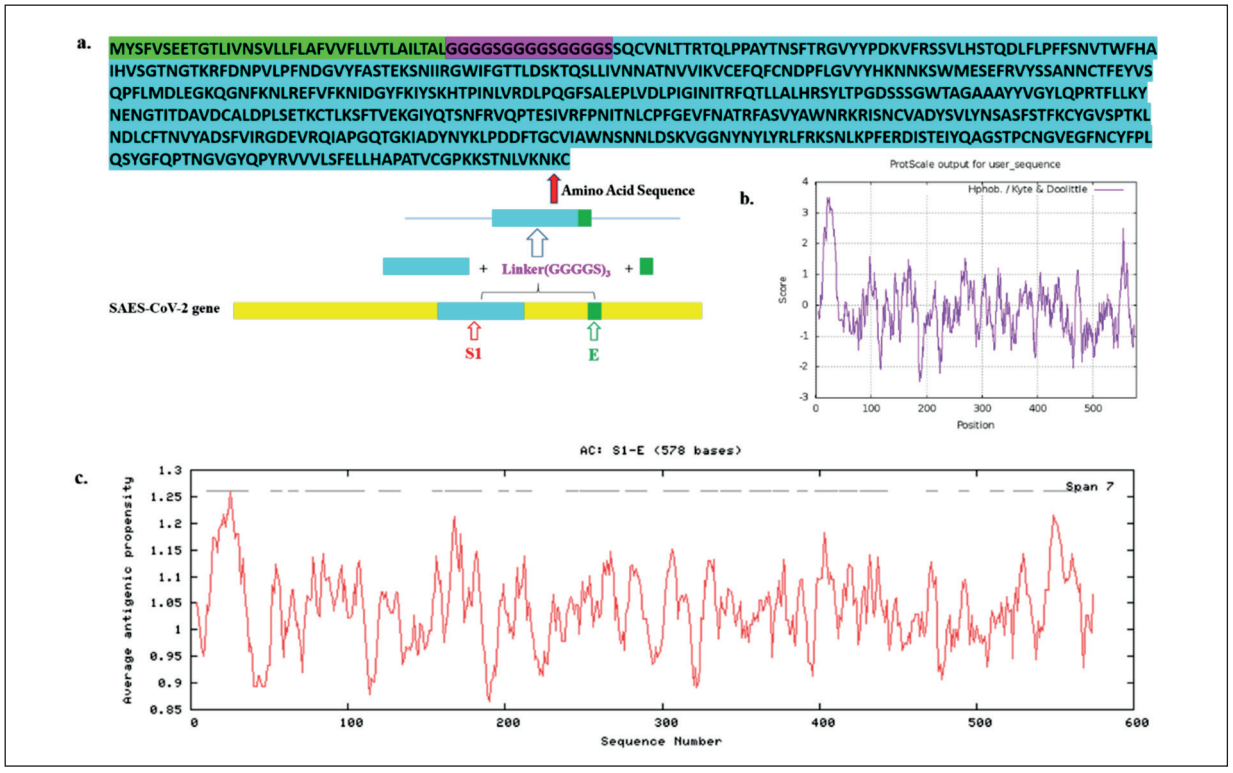

Figure 1. SARS-CoV-2. In the complete genome sequence of SARS-CoV-2, the gene sequences of the S1 subunit and the E protein were linked by a linker, and a pet-28a(+) recombinant plasmid containing the recombinant protein was constructed (**a**). Hydrophobicity analysis (**b**) and antigenic site prediction of the recombinant protein sequence (**c**)

duced by IPTG (0.5 mM) for 6 h, and this was followed by SDS-PAGE and staining with Coomassie Brilliant Blue (CCB) (Figure 2b). The recombinant SARS-CoV-2-S1-E protein was purified using an $Ni2^{+}$-charged resin column, and a band was detected at 55-70kDa (the estimated protein size was 65.5kDa) (Figure 2a/2b).

Characterization of the SARS-CoV-2 rS1-E-PLGA Nanovaccine

The rS1-E was emulsified with PLGA and lyophilised to obtain nanovaccines. SDS-PAGE and CCB staining were used to identify the integrity and correctness of the recombinant protein after PLGA coating and also prior to coating (Figure 2b). The nanoparticles were tested using various methods. The surface morphology of the nanovaccines was observed using scanning electron microscopy. The rS1-E-PLGA nanovaccines were spherical (Figure 3a). And the rS1-E proteins were granular and aggregated (Figure 3b). The release rate curve protein concentration at different time points was plotted using one-way ANOVA for the three test groups ($p > 0.05$) (Figure 3c). The release profile of S1-E-loaded PLGA exhibited a typical biphasic release pattern. The first stage was a rapid burst of release within the initial 12 h, and the release rate of the nanovaccine protein was 27.3%. A possible reason is that most of these proteins are attached to the surface of the nanovaccine or wrapped in the nanosurface protein. In the second stage, after 12 h the diffuse-driven S1-E was continuously released through the rigid PLGA core. At 48 h, the cumulative release rate of nanovaccine reached a peak of 68.41%. After 48 h, the cumulative release rate of the nanovaccine was slow; however, the release was complete. The nanoparticle size was 670±145 nm (Figure 3d). The LC and ER of rS1-E-PLGA were 8.8 and 63.6%, respectively. Furthermore, the zeta potential values of the nanovaccine were -22.3±6.89 (Figure 3e). To verify if the integrity of nanovaccines was affected during the production process, vaccine samples were assessed.

Evaluation of the Immune Effect of SARS-CoV-2 rS1-E-PLGA Nanovaccine in Mice

The mouse serum was collected at 0, 2, 4, 6, 8, and 10 weeks after immunisation (Figure 4a) that included immunisations for anti-S1 subunit antibody and anti-E protein antibody titres (Figure 4b/4c). The rS1-E vaccine group ($p < 0.05$) and S1-E-Freund's adjuvant group ($p < 0.05$) produced anti-S1 antibody. This indirectly indicates that the structure of our designed rS1-E did not change significantly after synthesis and expression. At 0-6 weeks after the initial immunisa-

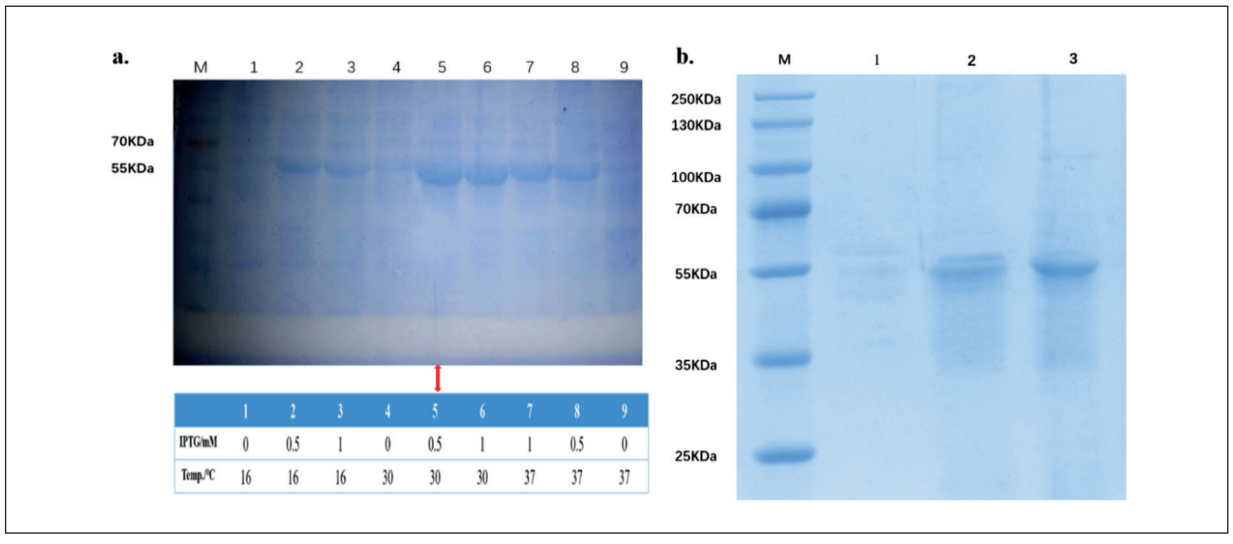

Figure 2. The rS1-E was expressed in Rosseta (DE3) cells at different temperatures and IPTG induction times, and the optimal IPTG concentration and expression temperature were determined to be 0.5 mM and 30°C (lane 5) (**a**). S1-E SDS-PAGE electrophoresis of S1-E recombinant protein supernatant after purification on nickel column is presented in lane 1, SDS-PAGE electrophoresis of inclusion bodies after purification on nickel column is presented in lane 2, and rS1-E-PLGA nanovaccine SDS-PAGE electrophoresis is presented in lane 3 (**b**).

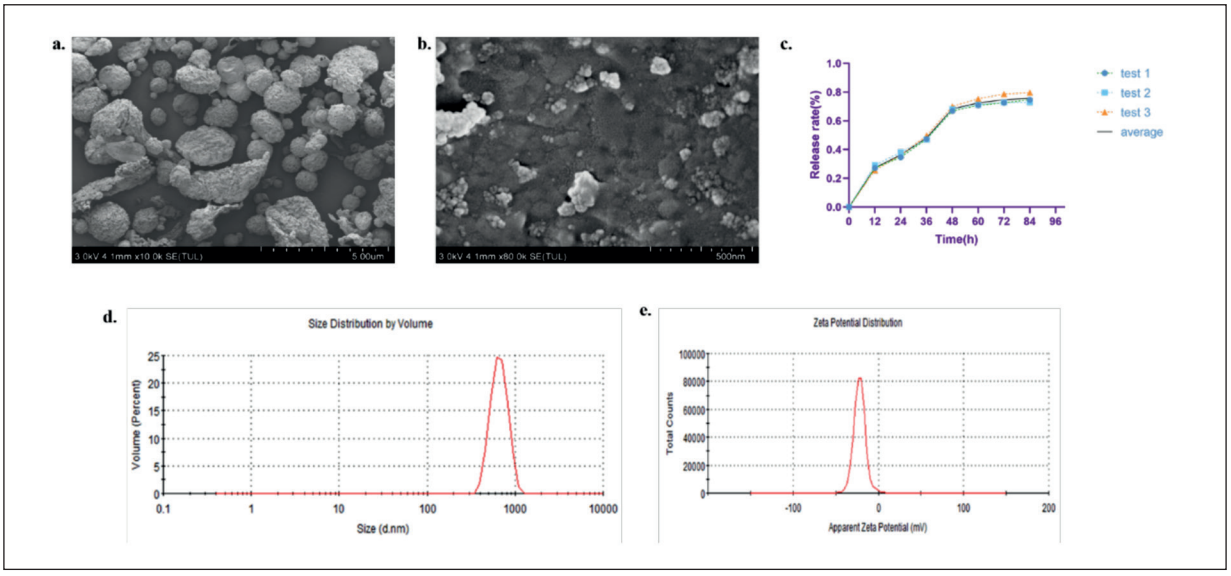

Figure 3. rS1-E-PLGA nanovaccine scanning electron microscope observation at 3.0kV 4.1 mm ×10.0k SE (**a**). rS1-E protein scanned by electron microscope at 3.0kV 4.1 mm × 80.0k SE (**b**). *In vitro* cumulative protein release from rS1-E-PLGA (**c**). Particle size distribution (**d**) and a Zeta potential (**e**) were also determined in the experiment.

tion, the anti-S1 and anti-E antibodies in mice exhibited a slowly increasing trend. After the 6th week (third vaccination), the anti-S1 and anti-E antibodies within the mice increased rapidly. At 8 weeks, the antibody titre in the mice no longer increased, and the antibody concentration in the mice reached a maximum.

The serum of mice immunised with rS1-E-PLGA nanovaccine for ELISA experiments was detected using anti-S1 antibodies and anti-E antibodies (Figure 4b/4c). It can be observed that the anti-S1 antibody titre and the anti-E antibody titre in the mouse serum were both significantly increased (p <0.05), and the upward trend is similar to that of the S1-E-Freund's adjuvant intramuscular injection (IM) group (p >0.05). The rS1-E-PLGA (IM) group exhibited a stronger titre (p <0.05) and higher antibody concentration (p <0.05) than did the S1-E (IM) group. The rS1-E-PLGA (IM) group concentration possessed a stronger titre (p <0.05) and a higher antibody concentration (p <0.05) than did the S1-E Freund's adjuvant (IM) group concentration. However, compared to that of the S1 group, the anti-S1 antibody level of the S1-E group was lower (p <0.05) (Figure 4b).

To determine the effectiveness of the antibodies produced by the mice after immunisation, we collected the mouse serum from the 10th week and incubated it with the pseudovirus carrying the S protein to test if the pseudovirus incubated with the serum could still infect HEK-293T/ACE2 cells. The results for rS1-E-PLGA (IM) and S1-E Freund's adjuvant (IM) group compared to those for the PBS-PLGA group indicated a significant increase (Figure 4d).

The weight of the mice was assessed regularly, and we observed that the weights of the mice that were vaccinated with various vaccines and of the PBS group all exhibited a trend for weight gain; the weights of each group were not significantly different (p >0.05) (Figure 4e).

At four weeks for the independent mouse S1-E group, rS1-E-PLGA group, and the S1-E Freund's adjuvant group, the spleen $CD4^+$ T cells and $CD8^+$ T cells exhibited significant changes in TNF-α and IL-2 levels (Figure 4f/g). Compared to the PBS-PLGA group, the other three groups exhibited a significant increase in TNF-α^+ $CD8^+$ T cells and $CD4^+$ T cells (%) (p <0.001) (Figure 4f). Compared to the PBS-PLGA group, the other three groups exhibited a significant increase in IL-2^+ $CD8^+$ T cells and $CD4^+$ T cells (%) ($p \leq 0.001$) (Figure 4g). However, endotoxins are unavoidable. These results may be enhanced by the presence of endotoxin that may act as an adjuvant to enhance the immune response in mice.

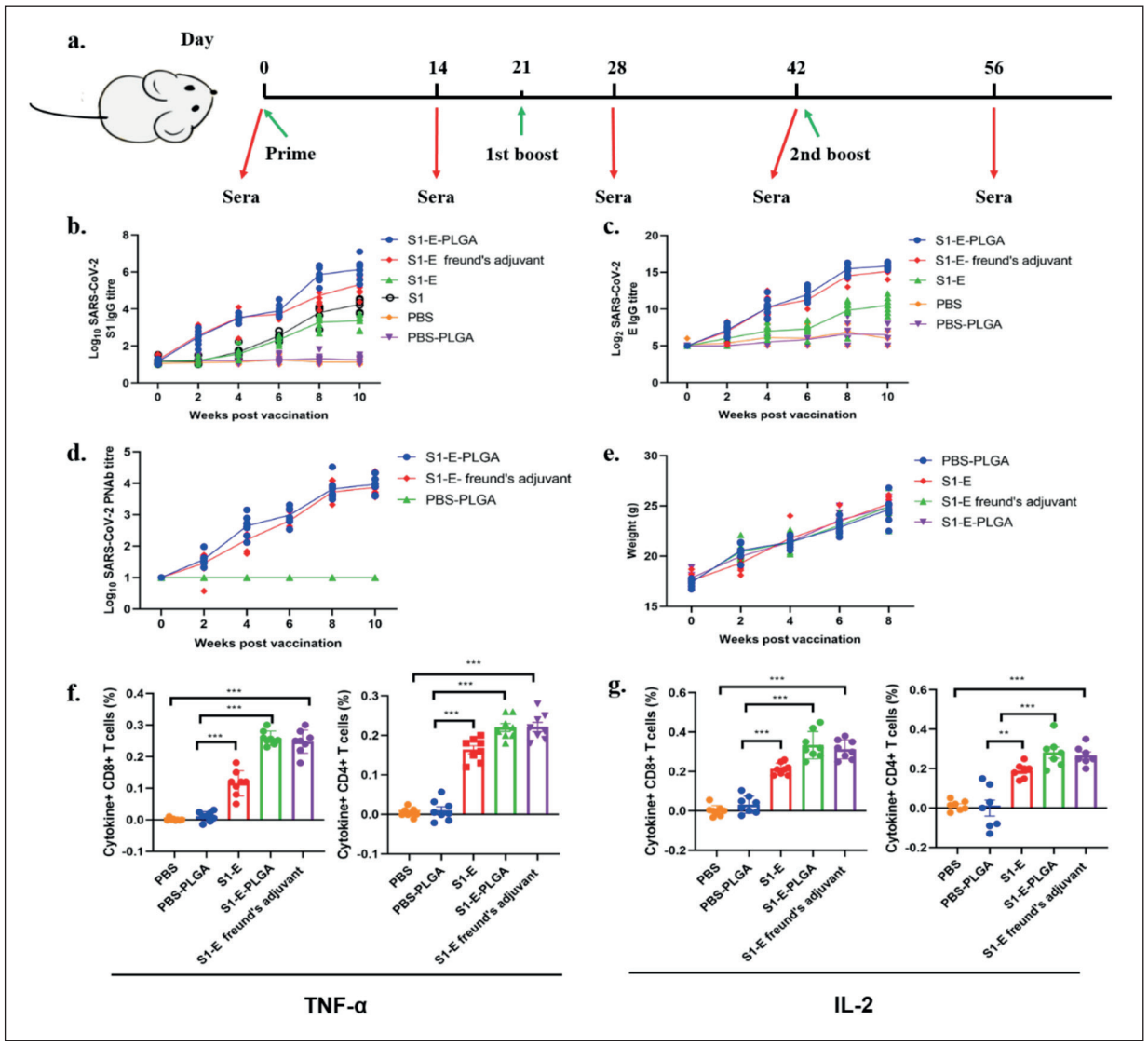

Figure 4. Timeline of vaccination and serum extractioxn in mice (**a**). At 0, 2, 4, 6, 8, and 10 weeks, mice sera were collected for ELISA to detect anti-S1 antibodies and anti-E antibodies (**b**, **c**). SARS-CoV-2 pseudovirus neutralization assay (**d**). Weight changes in mice after vaccination (**e**). Cellular immune responses were assessed at 4 weeks after vaccination using intracellular cytokine staining assays (TNF-α, **f**) (IL-2, **g**). Data are expressed as mean ± s.e.m. (p <0.05: *; p <0.01: **; p <0.001: ***).

Discussion

Compared with inactivated vaccines, The rS1-E-PLGA nanovaccine has different advantages. Although inactivated vaccine possesses many sites, it carries potential risks. Some antibodies can stimulate humans to produce invalid antibodies. Invalid antibodies cannot resist SARS-CoV-2 infection, and these antibodies may cause the immune system to produce excessive immune recognition that is harmful to health and can result in pathological conditions such as antibody-dependent enhancement and enhanced respiratory disease[14]. Parts of the structure have been used as antigens to avoid these undesired effects[15]. Similarly, we abandoned most of the structure of SARS-CoV-2 when designing the S1-E nanovaccine. During the process involving the use of rS1-E-PLGA nanovaccine to stimulate mice to produce antibodies, we observed the mental state of the mice and recorded their survival rate and weight changes. The mental state, body weight, and survival rate of vaccinated mice were not significantly different. The

survival rate was 100%. Compared to mRNA or adenovirus vector vaccines, rS1-E-PLGA nanovaccine may reduce the potential biological risk caused by nucleic acids.

The rS1-E-PLGA nanovaccine differs from the S1 subunit vaccine in that it contains more antigens in the E portion. The E is less immunogenic than is the S, but it is highly conserved. The rS1-E-PLGA nanovaccine contains both the immune target of the S1 subunit and the immune target of the envelope protein. No mutation sites on the E gene were observed in the Delta or Omicron strain. The development of a vaccine may provide a means for controlling the infection of the Delta and Omicron variant. As an adjuvant of the nanovaccine, the PLGA-loaded vaccine exhibits good stability, protects against premature antigen degradation, and facilitates improved antigen delivery to APCs. PLGA can also activate immune responses and induce cytokine production and antibody responses.

We produced the recombinant protein using a prokaryotic expression system that can express a large amount of protein to thus reduce costs (this is an important factor that should be considered when developing a vaccine candidate). The purpose was similar to that of van Oosten et al[16]. The rS1-E-PLGA nanovaccine is spherical under an electron microscope, and this is similar to the shape of other PLGA deliver drugs[17]. The zeta potential of the nanovaccines was negative, and the average value was less than -25 mV. This stable positively charged nanovaccine can prevent antigen degradation[18]. As an anionic nanovaccine, rS1-E-PLGA nanovaccines may promote efficient drug delivery. The large volume may induce a strong immune response[19]. Second, we investigated if the structure of the expressed protein was altered. When evaluating the immune effect of the vaccine, two antibodies were detected instead of anti-S1-E antibodies, as these can not only detect if the mouse has produced IgG after vaccination but also indirectly indicate that the protein structure expressed by the designed recombinant protein sequence has been significantly altered (immunocompetent domain still reserved). The neutralising antibody titre was used to test if the antibodies produced by vaccine stimulation were effective in preventing viral infection by infecting cells after co-incubation with SARS-CoV-2 in mouse serum. The results of the pseudovirus neutralising antibody test are similar to those of the neutralising antibody test[20]. The mouse serum was collected for pseudovirus neutralising antibody experiments, and higher neutralising antibody titres were obtained. This indicated that rS1-E-PLGA nanovaccine produced good and effective antibodies. Detection of the cytokines IL-2 and TNF-α in the spleen cells of vaccinated mice during the fourth week after immunisation indicated a higher cellular immune response. These results indicate that rS1-E-PLGA nanovaccine resulted in a good immune effect. In conclusion, we have developed rS1-E-PLGA nanovaccine. Neutralising antibodies and cytokines were produced after immunisation of mice. Next, we will conduct challenge experiments and clinical trials.

Conclusions

The variants of SARS-CoV-2 continue to emerge. The immunity of individuals to the new variants after other vaccinations is unknown. If a vaccine fails against a variant, it will take a long period of time to develop a vaccine or booster against that specific variant. The reassembly of nanoparticles with variant antigens enables the rapid production of target variant vaccines. Not only was rS1-E-PLGA capable of generating a strong immune response, but the manner in which the vaccine was designed could also be an effective means to rapidly respond to SARS-CoV-2 mutations and vaccine failure.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Ethics Statement

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