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Development of a Self-Emulsifying Adjuvant for Use in Swine Vaccines

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Abstract

Emulsion-based adjuvants are commonly used in animal vaccine formulations for several reasons including affordability, stability, and efficacy in inducing disease-protecting immune responses. Here we report a novel, cost-effective, stable, self-emulsifying adjuvant (SEA1) that is prepared by a simple low shear process or low-energy mixing without the use of expensive and complex proprietary equipment. Characterization of the SEA1 adjuvant showed good stability at different temperatures (4°C, 20°C, and 37°C) after one month of storage. Minimal changes in droplet size distribution, polydispersity index, Zeta potential and pH in 1-month-old SEA1 preparations were observed when compared with a fresh SEA1 preparation. SEA1 emulsion-based experimental vaccine preparations effectively stimulated humoral immunoglobulin (IgG) responses in mice and swine and were comparable to commercially available adjuvants Montanide ISA 201 and 206.

Keywords

swine, vaccine, adjuvant, classical swine fever

Cover Page Footnote

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Summary

Emulsion-based adjuvants are commonly used in animal vaccine formulations for several reasons including affordability, stability, and efficacy in inducing disease-protecting immune responses. Here we report a novel, cost-effective, stable, self-emulsifying adjuvant (SEA1) that is prepared by a simple low shear process or low-energy mixing without the use of expensive and complex proprietary equipment. Characterization of the SEA1 adjuvant showed good stability at different temperatures (4°C, 20°C, and 37°C) after one month of storage. Minimal changes in droplet size distribution, polydispersity index, Zeta potential and pH in 1-month-old SEA1 preparations were observed when compared with a fresh SEA1 preparation. SEA1 emulsion-based experimental vaccine preparations effectively stimulated humoral immunoglobulin (IgG) responses in mice and swine and were comparable to commercially available adjuvants Montanide ISA 201 and 206.

Introduction

Adjuvants are vaccine additives that potentiate the effectivity of vaccines. Common adjuvants used in human and animal vaccines are emulsion-based adjuvants.[2,3] Emulsions are composed of two immiscible phases, such as oil and an aqueous phase, that are stabilized into a distinct continuous phase by an interfacial surfactant layer.[4] Surfactants, also known as emulsifiers, act as a "bridge" between the immiscible phases by virtue of the dual nature of their molecular structure, which reduces the forces between liquid molecules and form emulsions.

Subunit vaccines have two main components—adjuvants and antigens. Antigens typically consist of proteins derived from the pathogen, against which a protective immune

[1] Department of Anatomy and Physiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS. This research is supported by awards from the National Bio and Agro-Defense Facility Transition Fund, the USDA National Institute of Food and Agriculture, Hatch-Multistate project, grant number [1021491]; USDA ARS Non-Assistance Cooperative Agreements, grant numbers [58-8064-8-011, 58-8064-9-007, 58-3020-9-020, 59-0208-9-222]; USDA-FAS-10960-0700; USDA NIFA Award #2022-67015-36516 and USDA NIFA Subaward #25-6226-0633-002; National Pork Board Grant, grant number [18-059].

[2] Madera R, Burakova Y, Shi J (2022) Emulsion Adjuvants for Use in Veterinary Vaccines. Methods Mol Biol 2412:247–253. https://doi.org/10.1007/978-1-0716-1892-9_11.

[3] Fox CB, Haensler J (2013) An update on safety and immunogenicity of vaccines containing emulsion-based adjuvants. Expert Rev. Vaccines.

[4] Lee S, Nguyen MT (2015) Recent Advances of Vaccine Adjuvants for Infectious Diseases. Immune Netw 15:51. https://doi.org/10.4110/in.2015.15.2.51.

response is desired. In emulsion-based vaccine formulations, the aqueous phase contains antigens that confer protection against diseases.

The design of emulsions for adjuvant development involves several parameters. Of importance is the nature and number of components[5] that greatly impact stability and efficacy. Emulsion adjuvants contribute towards antigen dose sparing and enhanced antibody titers[6] that are very beneficial in the development of vaccines for the livestock industry. Other important aspects of emulsion adjuvants include cost effectiveness, ease of use, storage convenience and minimal impact on animal growth.[7]

In the present study, we reported on SEA1, a novel, emulsion-based adjuvant that is stable, economical, easily prepared without utilizing specialized equipment, and effective in eliciting antibody immune responses in mice and swine.

Procedures

Antigens and adjuvants for immunization

Ovalbumin (EndoFit OVA, Invivogen, San Diego, CA), the main component of the chicken egg white, served as antigen in the mouse studies. The OVA antigen was diluted in sterile endotoxin-free phosphate-buffered saline (PBS; Gibco, Fisher Scientific, USA) and administered at 100 μg per dose. Insect cells were used to express recombinant classical swine fever (CSF) surface E2 protein which was served as antigen in swine studies. The CSF E2 was prepared as described in a previous study,[8] diluted in sterile endotoxin-free PBS, and administered at 50 μg per dose in pig immunization studies. Commercial adjuvants Montanide ISA 201 and 206 (Seppic, France) emulsions were prepared according to manufacturer's instructions. Briefly, the adjuvant and aqueous antigen solution were warmed to 31°C in a water bath. The antigenic solution was added to the adjuvant at a 1:1 volume ratio and stirred at room temperature with a sterile magnetic bar for 5 min at 350 rpm. The vaccines were kept at room temperature for 1 hour before immunization.

Preparation of SEA1 adjuvant and formulation of emulsions

The SEA1 is composed of three components: mineral oil Drakeol 5 (Calumet Penreco LLC, Karns City, PA) and two surfactants—a nonionic Tween surfactant (MP Biomedicals, Solon, OH) and a proprietary polymeric surfactant. SEA1 adjuvant was prepared by mixing mineral oil with surfactants using a sterile magnetic stirrer bar at 500 rpm for 5 hours at room temperature. SEA1 preparations were stored in the dark at room temperature until use. SEA1 emulsions were formed by pre-warming both adjuvant and antigen solutions at 37°C for 10 minutes. The antigen solution (2:1 or 1:1 antigen to adjuvant volume ratio) was added slowly to SEA1 adjuvant while being

[5] Burakova Y, Madera R, McVey S, et al (2018) Adjuvants for Animal Vaccines. Viral Immunol 31:11–22. https://doi.org/10.1089/vim.2017.0049.

[6] Lee S, Nguyen MT (2015) Recent Advances of Vaccine Adjuvants for Infectious Diseases. Immune Netw 15:51. https://doi.org/10.4110/in.2015.15.2.51.

[7] Madera R, Burakova Y, Shi J (2022) Emulsion Adjuvants for Use in Veterinary Vaccines. Methods Mol Biol 2412:247–253. https://doi.org/10.1007/978-1-0716-1892-9_11.

[8] Madera R, Gong W, Wang L, et al (2016) Pigs immunized with a novel E2 subunit vaccine are protected from subgenotype heterologous classical swine fever virus challenge. BMC Vet Res 12:1–10. https://doi.org/10.1186/s12917-016-0823-4.

stirred with a magnetic bar (500 rpm) at room temperature. The emulsions were stirred for an additional 30 to 60 minutes before use.

Physical characterization and stability

Droplet size, polydispersity, and zeta potential of the emulsions were determined by dynamic light scattering using a Zetasizer Nano ZS 90 (Malvern Instruments, Westborough, MA) with a 633 nm He-Ne laser at a scattering angle of 173°. Each sample was diluted by a factor of 100 with PBS for droplet size analyses, and with a 0.001 M sodium solution for Zeta potential measurements to prevent multiple scattering effects. Measurements were taken in triplicate with results reported as mean of three measurements. Emulsions were imaged with transmission electron microscopy (TEM) using the FEI CM100 electron microscope at the Kansas State University Division of Biology microscopy facility.

An accelerated stability test or centrifuge test was performed to evaluate and predict stability. Emulsions were centrifuged at 2,500 rcf at 20°C for 15 minutes and examined for the presence of creaming, sedimentation, and phase separation.

Temperature variation testing was carried out to evaluate emulsion stability. The emulsions were stored at 4°C (in a refrigerator), 20°C (at room temperature), and 37°C (in an oven) for one month. Visual tests were performed periodically. The samples were considered to pass the visual test in the absence of creaming or phase separation. All emulsion preparations from different conditions were subjected to droplet size, polydispersity, and zeta potential determination, as well as pH determination of emulsion samples. Tests were carried out in triplicate.

Optimization process of SEA1 emulsion

Process parameters were investigated to determine the optimal SEA1 adjuvant preparation. The duration of mixing for emulsion formation and the temperature of SEA1 and antigen solution at time of mixing were determined. Different mixing speeds were used during the SEA1 emulsion formation to determine the effect on droplet size. The impact of antigen diluent nature (water or PBS) on droplet size distribution was also determined.

Safety study in mice

A total of 25 female BALB/c mice (Charles River Laboratories International) weighing 15 to 20 g were randomly assigned into five groups (n = 5 for each group). Four groups of mice were injected intraperitoneally or subcutaneously at 1 mL volume with either PBS or 100 μg OVA in SEA1 vaccine preparations. A control group received PBS only. Mice were closely monitored at least twice daily for 7 consecutive days. This study was conducted following the approval of the Institutional Animal Care and Use Committee (IACUC) of Kansas State University (IACUC 4121).

Adjuvanticity studies in mice and swine

The adjuvanticity of SEA1 was next tested with the OVA antigen in mice and the CSF E2 antigen in swine. Seven-to-nine-week-old female BALB/c mice (n = 5 for each group) were immunized subcutaneously (100 ml volume) with one dose of SEA1 vaccine preparation containing 100 mg of OVA antigen. SEA1 adjuvant and antigen

volume ratio was 1:2. Blood was collected at 14, 21, and 28 days post vaccination. Sera was separated from the blood by centrifugation and stored at -20°C until use.

Three-week old conventional Large White-Duroc crossbred weaned, specific-pathogen free, female piglets (n = 5 for each group) were purchased from a commercial vendor. The pigs were fed with a standard commercial diet and housed at the Large Animal Research Center (LARC) at Kansas State University. Pigs were immunized intramuscularly (2 mL volume) with one or two doses of CSF E2 antigen (50 mg per dose) with SEA1 adjuvant, with second dose given at 21 days after initial immunization. Injection sites were observed for swelling, erythema, abscess, and induration. Serum was collected weekly for 35 days. All animal studies were conducted in accordance with approved IACUC protocols. Serum samples from mouse and swine studies were analyzed for OVA-specific and E2-specific total immunoglobulin (IgG) using ELISA. ELISA was performed as described in a previous study.[9]

Results and Discussion

The focus of this work was to develop a low-cost, stable, and effective adjuvant that is suitable for use in swine vaccines. Self-emulsification is sensitive to the ratio of oil and surfactants added to the aqueous phase.[10] After numerous vaccine formulations and screening, we report the development of a self-emulsifying, mineral-oil-based SEA1 adjuvant. Upon preparation, the appearance of SEA1 is a clear yellow liquid (Figure 1a) that turns into a milky-white liquid upon addition of aqueous solution (Figure 1b). The TEM imaging showed the formation of multiple water-in-oil-in-water (W/O/W) structures (Figure 1c). The W/O/W adjuvants has been shown to have stability and to induce adequate vaccine potency.[11]

Physical characterization of SEA1

Droplet size of emulsion adjuvants has a significant impact on their potency. The droplet size of freshly prepared SEA1 was 74.61 nm. It has been shown that immune cells take up and process particles more efficiently in the nanoscale range, leading to more robust adaptive immune responses.[12,13] In addition, emulsions with droplet sizes less than 200 nm could be sterilized by membrane filtration, without the need for autoclaving, which is damaging for heat-sensitive formulations.

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[9] Madera RF, Wang L, Gong W, et al (2018) Toward the development of a one-dose classical swine fever subunit vaccine: Antigen titration, immunity onset, and duration of immunity. J Vet Sci 19:393–405. https://doi.org/10.4142/jvs.2018.19.3.393.

[10] Shah RR, Dodd S, Schaefer M, et al (2015) The Development of Self-Emulsifying Oil-in-Water Emulsion Adjuvant and an Evaluation of the Impact of Droplet Size on Performance. J Pharm Sci 104:1352–1361. https://doi.org/10.1002/jps.24337.

[11] Leclercq SY, Dos Santos RMM, MacEdo LB, et al (2011) Evaluation of water-in-oil-in-water multiple emulsion and microemulsion as potential adjuvants for immunization with rabies antigen. Eur J Pharm Sci 43:378–385. https://doi.org/10.1016/j.ejps.2011.05.008.

[12] Shah RR, Taccone M, Monaci E, et al (2019) The droplet size of emulsion adjuvants has significant impact on their potency, due to differences in immune cell-recruitment and -activation. Sci Rep 9:1–9. https://doi.org/10.1038/s41598-019-47885-z.

[13] Kanchan V, Panda AK (2007) Interactions of antigen-loaded polylactide particles with macrophages and their correlation with the immune response. Biomaterials 28:5344–5357. https://doi.org/10.1016/j.biomaterials.2007.08.015.

Slight changes in droplet sizes were observed after one month storage at 4°C, 20°C, and 37°C (Table 1). A more notable increase in mean droplet size was observed in SEA1 stored at 4°C (Figure 2a). Droplet size distribution was compared to W/O/W adjuvant ISA 206. The droplet size of SEA1 was 74.61 nm and smaller than ISA 206 (294 nm, Figure 2b).

It has been demonstrated that lower polydispersity index (PDI) of colloidal systems signifies higher stability.[14] SEA1 had very low PDI indicating a stable and homogenous formulation. SEA1 stored at 4°C for one month showed higher PDI. The change in PDI for SEA1 at 20°C and 37°C was minimal (Table 1). No significant changes were observed in Zeta potential and pH in all the samples (Table 1). Taken together, these results indicate SEA1 is a stable adjuvant for at least a month when stored at 20°C and 37°C.

Evaluation of stability by centrifuge test

The centrifuge test involves the use of centrifugal force to assess stability and to predict the shelf life of emulsions.[15] SEA1 emulsion preparations were centrifuged at 2,500 rcf for 15 minutes at 20°C. There was no creaming, sedimentation, and phase separation observed after centrifugation. The results indicate SEA1 emulsion stability even in the presence of centrifugal force.

Process optimization of SEA1

The impact of four emulsification parameters (time of mixing, initial temperature of components, speed of mixing, and nature of aqueous phase) on the droplet size and polydispersity of SEA1 emulsions were investigated (Figure 3). Longer duration of mixing resulted in lower mean droplet size and smaller PDI (Figure 3a). SEA1 emulsions prepared at higher temperatures resulted in lower mean droplet size and smaller PDI (Figure 3b). SEA1 emulsions prepared with low-shear mixing resulted in lower mean droplet size and smaller PDI (Figure 3c). SEA1 emulsions prepared with PBS or water had almost identical size distributions (Figure 3d). The results indicate that duration of mixing, initial temperature of components upon mixing, and speed of mixing influenced droplet size and PDI. The use of PBS or water in the aqueous phase appears to have little or no effect on droplet size and PDI. These parameters are not only important indicators of the physical stability of SEA1, but also have a great impact on the efficacy of the SEA1 emulsions as delivery agents in vaccines.

Safety evaluation in mice

A substantial amount (1 mL) of SEA1 injected into mice either intraperitoneally or subcutaneously did not cause mortality in all SEA1-injected mice. Other than an oily appearance in some mice on the first day post injection, no behavioral differences were observed compared to the control group. No swelling, erythema, abscess, or induration in the injection sites, and no adverse reactions were observed. Except for one mouse injected intraperitoneally, all mice gained weight after 7 days post injection (data not shown). Hence, the results indicate SEA1 is safe to use in animals with no observed adverse reactions.

[14] Djerdjev AM, Beattie JK (2008) Enhancement of ostwald ripening by depletion flocculation. Langmuir 24:7711–7717. https://doi.org/10.1021/la800140s.

[15] Teh SS, Mah SH (2018) Stability evaluations of different types of vegetable oil-based emulsions. J Oleo Sci 67:1381–1387. https://doi.org/10.5650/JOS.ESS18067.

Adjuvanticity studies to establish SEA1 adjuvant potency

The conventional OVA immunization mouse model was performed for initial proof of concept. Mice injected with one dose of the SEA1 vaccine formulation showed an increase in OVA-specific antibodies (Figure 4). Although slightly lower than results with ISA 206, the results indicate SEA1 induced antigen specific antibodies and was comparable to a commercially available adjuvant.

The SEA1 adjuvant activity was then tested in swine. Pigs immunized with one dose and two doses of SEA1 vaccine formulation with CSF E2 as antigen resulted in an increase in E2-specific antibodies (Figure 5). The elicited antibodies were comparable to results with ISA 201 and a novel adjuvant OW14 previously shown to induce robust immune responses in pigs.[16]

Our results have demonstrated that SEA1 is a stable, safe, cost-effective, easily produced, self-emulsifying adjuvant. Further evaluation of the SEA1 adjuvant in additional animal studies will shed light on its efficacy and potential use in swine vaccines.

Table 1. Physical characterization of an SEA1 emulsion right after mixing and one month post emulsification

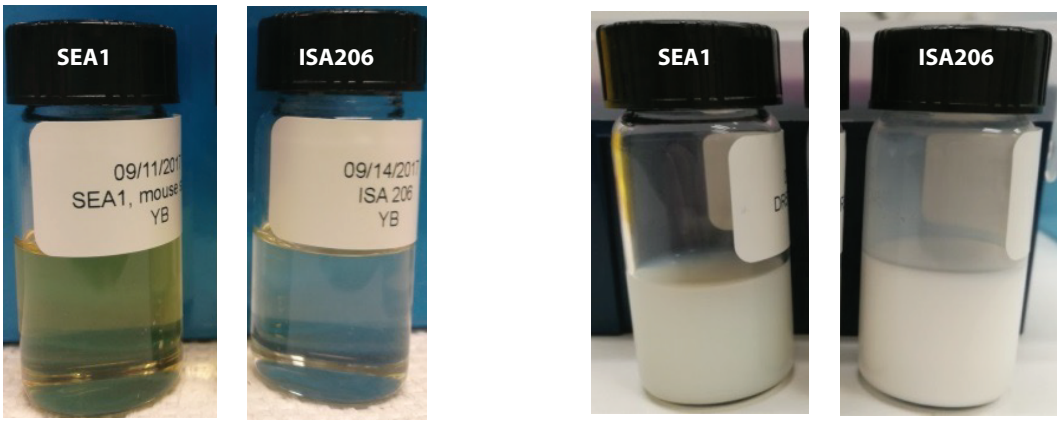
Physical characteristics of SEA1	Freshly prepared	1 month later
Mean droplet size (nm), ± SD	74.61 nm ± 0.81	83.80 nm ± 0.81 (4°C) 74.96 nm ± 0.59 (20°C) 79.42 nm ± 0.29 (37°C)
Polydispersity index (PDI) ± SD	0.049 ± 0.004	0.177 ± 0.025 (4°C) 0.047 ± 0.015 (20°) 0.044 ± 0.025 (37°C)
Zeta potential (mV) ± SD	-23.67 ± 1.44	-24.00 ± 1.28 (4°C) -23.10 ± 0.26 (20°C) -25.40 ± 1.47 (37°C)
pH	6.67	6.64 (4°) 6.68 (20°C) 6.64 (37°C)

The SEA1 was analyzed for droplet size, polydispersity index, Zeta potential and pH. Values are expressed as mean ± standard deviation, n = 3.

[16] Galliher-Beckley A, Pappan LK, Madera R, et al (2015) Characterization of a novel oil-in-water emulsion adjuvant for swine influenza virus and *Mycoplasma hyopneumoniae* vaccines. Vaccine 33:2903–2908. https://doi.org/10.1016/j.vaccine.2015.04.065.

a. Visual appearance of SEA1 and ISA206 adjuvants

b. Visual appearance of SEA1 and ISA206 emulsions after mixing with PBS (1 adjuvant : 2 PBS volume ratio)



c.

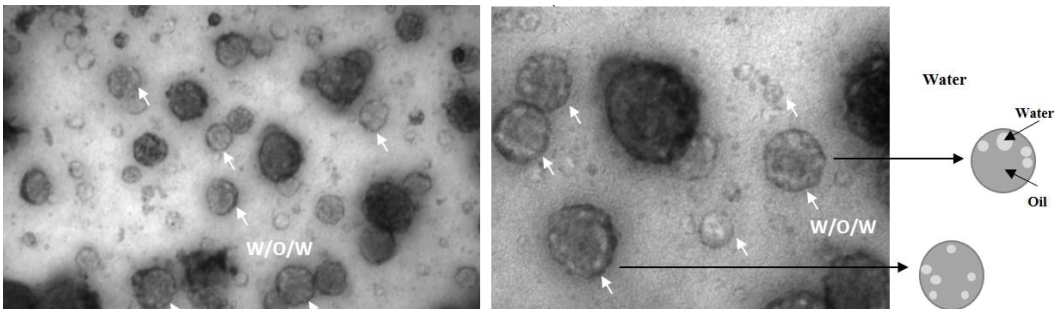
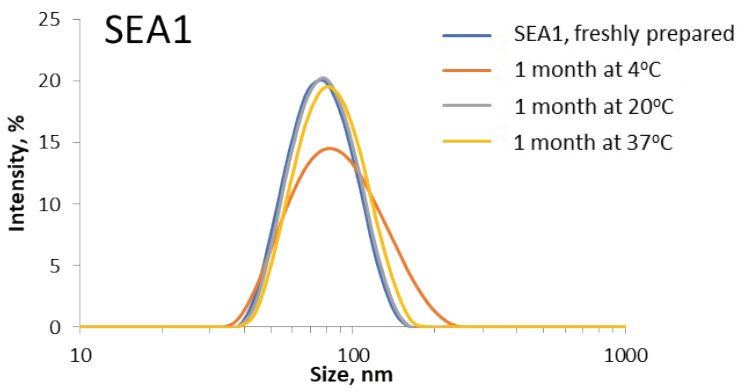


Figure 1. Visual appearance of SEA1 as compared to Montanide ISA 206 (a), and the formation of milky-white emulsions after mixing adjuvants with aqueous solution such as phosphate-buffered saline (PBS) (b). Transmission electron microscope (TEM) images of emulsion prepared with SEA1 adjuvant. Droplets with multiple water-in-oil-in-water structures were observed (c).

a. Change in droplet size distribution after 1 month storage at different temperatures



b. Droplet size distribution of emulsion-based vaccines

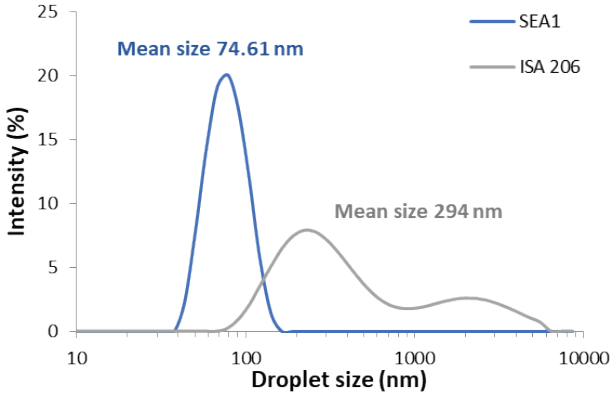


Figure 2. Droplet size distribution after one month storage at different temperatures as compared to freshly prepared SEA1 emulsion (a). Slight increase in mean droplet size was observed in emulsion sample stored at 4°C. Droplet size distribution of freshly prepared SEA1 and ISA 206 emulsions (b).

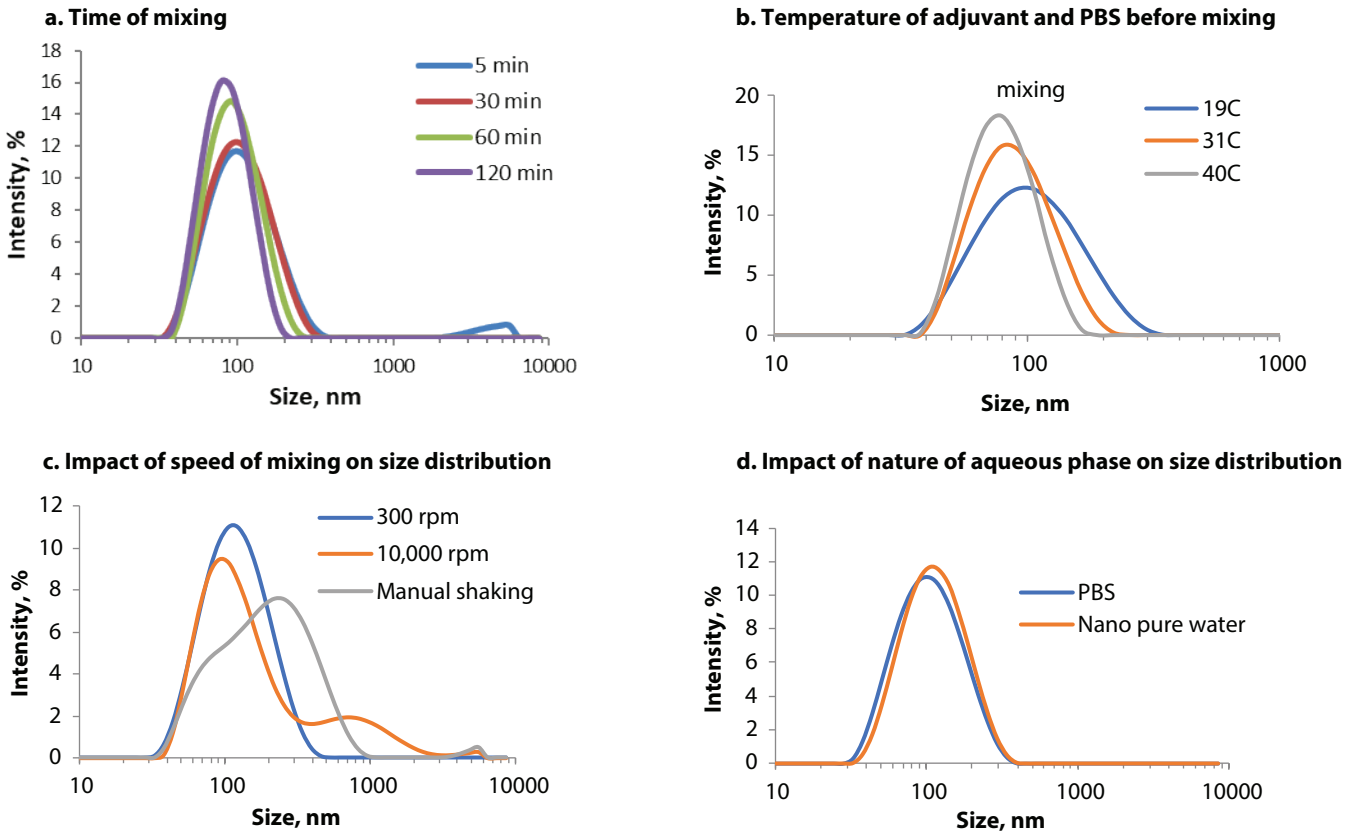


Figure 3. Optimization process of an SEA1 emulsion. SEA1 emulsions prepared at different times of mixing (a). Longer duration of mixing resulted in lower mean droplet size and smaller polydispersity index (PDI). SEA1 emulsions prepared at various temperatures of adjuvant and aqueous solution before mixing (b). Higher temperatures resulted in lower mean droplet size and smaller PDI. SEA1 emulsions prepared at different speeds of mixing (c). Smaller droplets were observed at lower shear mixing. SEA1 emulsions prepared with phosphate-buffered saline (PBS) and water had almost identical size distribution (d).

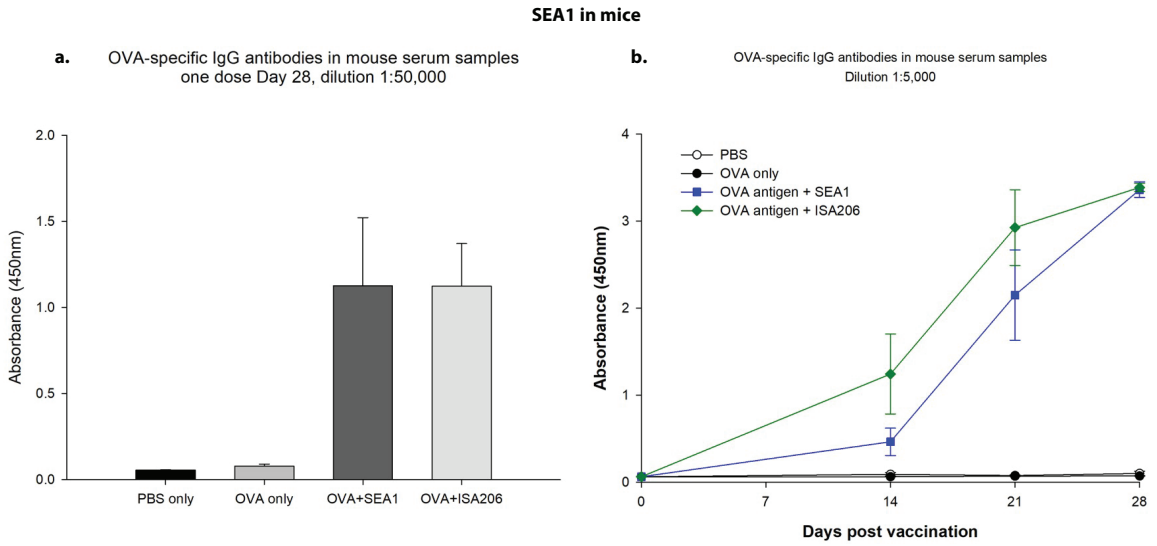


Figure 4. SEA1 adjuvant evaluation in mice. Mice (n = 5) were immunized with one dose of ovalbumin protein (OVA)-SEA1 emulsion, with phosphate-buffered saline (PBS) and OVA only as negative controls. OVA-specific antibodies were determined by ELISA assay. A group of mice were also immunized with OVA-ISA 206 emulsion as positive control (a). OVA-specific antibodies elicited were determined for 4 weeks post immunization (b).

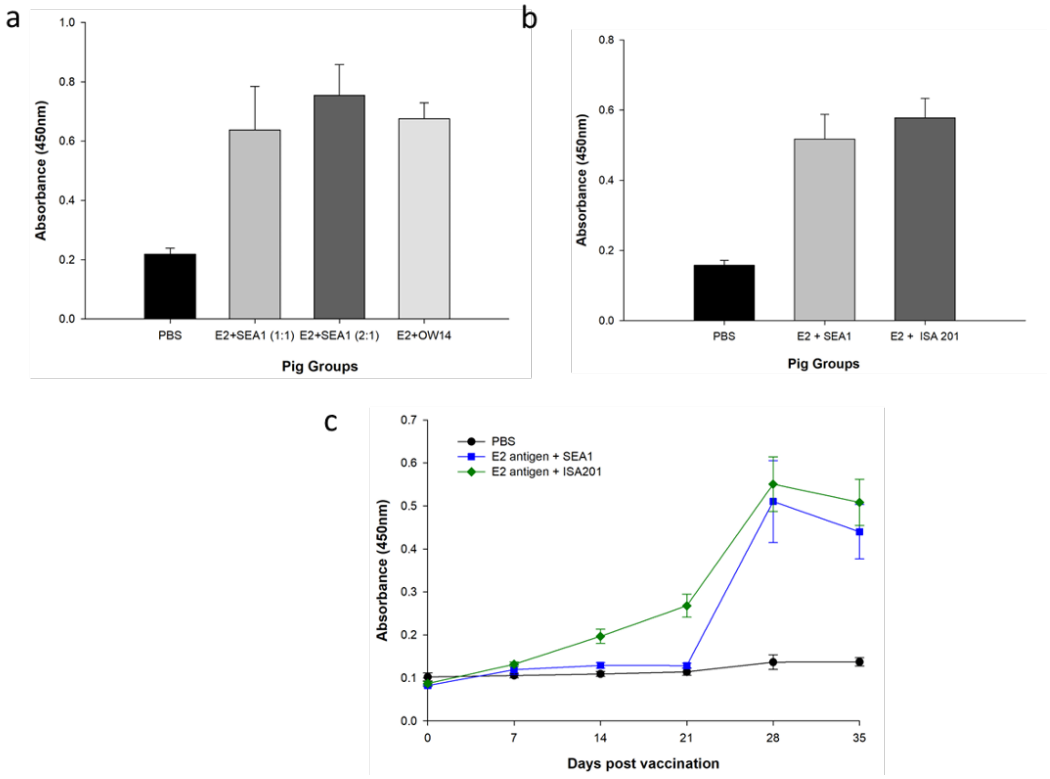


Figure 5. SEA1 adjuvant evaluation in swine. Pigs (n = 5) were immunized with one dose of classical swine fever virus E2 glycoprotein (E2) and phosphate buffered saline (PBS) as negative control. E2-specific antibodies were determined by ELISA and compared with pigs immunized using an oil-water adjuvant OW14 (also developed by our research group) (a). In another experiment, pigs were immunized with two doses of E2. Dose 2 was given at three weeks after the first dose. E2-specific antibodies were compared with pigs immunized using ISA 201 adjuvant (b). The E2-specific antibodies in serum were determined weekly for 7 weeks post initial immunization (c). Antibody titers were comparable between SEA1 and established adjuvants OW14 and ISA201.