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Novel nanoemulsion adjuvant stabilized by TPGS possesses equivalent physicochemical properties, Turbiscan stability, and adjuvanticity to AS03 for eliciting robust immunogenicity of subunit vaccines in mice

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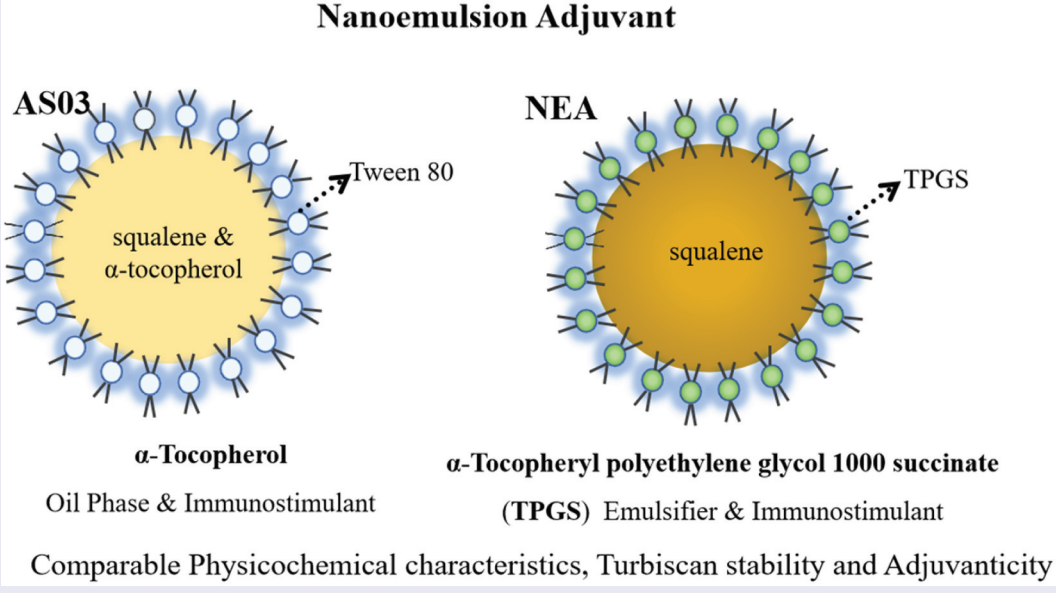
ABSTRACT

Emulsion-based antigen delivery systems have emerged as a novel approach to enhance the effectiveness of subunit vaccines. This study presents the development of a newly formulated oil-in-water (o/w) nanoemulsion adjuvant (NEA) composed of squalene oil and α-tocopheryl polyethylene glycol 1000 succinate (TPGS), which serves dual roles as an emulsifier and an immunostimulator. In comparison to AS03, an FDA-approved emulsion adjuvant that includes α-tocopherol, squalene, and polysorbate 80, NEA is devoid of α-tocopherol and exhibits comparable physicochemical properties, including particle size, polydispersity index, morphology, pH, zeta potential, and viscosity. Stability assessments conducted using Turbiscan Lab indicated that NEA undergoes an uplift process without experiencing flocculation, agglomeration or delamination. Model subunit antigens of recombinant glycoprotein E (gE) targeting the varicella-zoster virus (VZV) and highly purified hemagglutinin (HA) protein against trivalent seasonal influenza viruses (TIV) were employed to assess the adjuvanticity of NEA. It was revealed that the specific anti-gE IgG titers induced by the gE/NEA were markedly higher than those generated by gE alone, with titers of 13,000 *vs* 3,000 for the primary vaccination, and 5×10^6 *vs* 5×10^4 for the booster vaccination. Additionally, the TIV/NEA group exhibited a significantly improved immunogenic response relative to TIV alone across all three HA antigens at six-week after immunization, as evidenced by anti-HA titers of 256 *vs* 32. Furthermore, the NEA demonstrated no significant difference in efficacy compared to AS03 in both the VZV and TIV vaccines. Consequently, NEA presents a promising alternative to AS03 for the development of effective subunit vaccines.



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Introduction

Subunit vaccines, comprising highly recombinant or purified protein antigens designed to induce protective immunity, demonstrate advantages such as superior safety profiles, lower costs of goods, stability, and manufacturing scalability.[1] However, subunit vaccines exhibit poor immunogenicity and may be unsuitable for individuals with compromised immunity, particularly young children and the elderly. Adjuvant

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systems have been demonstrated to enhance the immunogenicity of subunit vaccines by preventing antigen degradation and facilitating antigen delivery to antigen-presenting cells.[2–4] The utilization of adjuvants is effective in reducing the required antigen dose and the number of vaccinations necessary to elicit the desired immune response, which is crucial for addressing pandemics and enhancing global vaccine supply.[5–7]

At present, adjuvants comprise various components, including aluminum salts,[8,9] emulsions such as MF59 (Novartis Vaccines and Diagnostics)[10,11] and AS03 (GlaxoSmithKline Biologicals),[12,13] AF03 (Sanofi Paster),[14] toll-like receptor (TLR) agonists (such as CpG oligodeoxynucleotides),[15] monophosphoryl lipid A adsorbed on aluminum salts as in AS04,[16] or combinations of immunopotentiators (QS-21 and MPL in AS01).[17] Among these, numerous clinical trials have demonstrated that oil-in-water (o/w) emulsion adjuvants (MF59, AS03, and AF03) exhibited superior performance compared to aluminum salts in enhancing vaccine immunogenicity without inducing unacceptable adverse reactions.[3]

AS03 comprises squalene and α-tocopherol stabilized by Tween 80 in an o/w emulsion system, which has been utilized in the pandemic influenza A (H1N1) pdm09 vaccines, Pandemrix® and its identical sister vaccine, Arepanrix® in Europe.[18] The inclusion of α-tocopherol as an immunostimulant distinguishes AS03 from other emulsion adjuvants employed in human vaccines.[19] Research has demonstrated that AS03 enhances the vaccine antigen-specific adaptive response by activating the innate immune system locally and by increasing antigen uptake and presentation in draining lymph nodes, a process that is likely modulated by the presence of α-tocopherol.[12,13,18,20] However, the European Medicines Agency (EMA) has recently recommended restricting the use of Pandemrix in individuals under 20 years of age, following a potential association with very rare cases of narcolepsy in young children in Scandinavia.[21] This phenomenon necessitates further investigation, as a confirmed link to the adjuvant has yet to be established. The development of an alternative to AS03 is motivated by various factors that extend beyond patent and compositional limitations. These factors include the diversification of supply chains, the management of costs, the adaptation to various vaccine platforms, the optimization of immune responses, the need to address emerging infectious diseases, the challenge of drug resistance, and the promotion of technological innovation. Developing alternatives to the AS03 adjuvant not only helps improve the accessibility and safety of vaccines but also promotes technological innovation and enhances the global capacity to respond to infectious diseases.

α-tocopherol is one of the eight isoforms of vitamin E that is most widely distributed in nature.[22] Through the prevention of membrane lipid oxidation, vitamin E contributes to the maintenance of cell membrane integrity, gene expression, and intracellular signal transduction.[23–28] Research has suggested that vitamin E dietary supplementation possesses immunomodulatory properties, such as counteracting age-related impairments of naive T-cell activation and age-related overproduction of prostaglandin E2 by macrophages, and may also have the capacity to restore age-related impairments in blood neutrophil, lymphocyte, and natural killer cell functions.[29,30] The detoxification of reactive oxygen radicals at injection sites, which are produced during immune cell processing and presentation of antigens, is one of the advantageous qualities of vitamin E in adjuvant systems.[23] In addition to α-tocopherol, other vitamin E derivatives, including α-tocopheryl acetate, α-tocopheryl nicotinate, and α-tocopheryl succinate, have been particularly encouraged in the development of various veterinary vaccines,[24,31–35] given their ability to induce differentiation, inhibit proliferation, and promote apoptosis in cancer cells.[31] For instance, α-tocopheryl acetate has successfully replaced the mineral oil component of Freund's adjuvant in a vaccine for chickens.[35] Furthermore, it was observed that incorporating α-tocopheryl acetate into vaccines resulted in improved specific immune responses compared with conventional adjuvants in sheep.[24]

As a derivative of vitamin E, α-tocopheryl polyethylene glycol 1000 succinate (TPGS) has exhibited considerable potential as a novel adjuvant, either independently or in conjunction with an appropriate delivery system.[36–38] For example, the immune response elicited by diphtheria toxoid-loaded PCL-TPGS microspheres following nasal administration was significantly greater than that achieved with PCL microspheres alone.[37] The inclusion of TPGS was found to enhance the antibody responses in oral vaccine preparations targeting the Vibrio anguillarum O2 antigen.[39] This water-soluble TPGS is synthesized through the esterification of vitamin E succinate with polyethylene glycol (PEG) 1000. Its amphipathic characteristics facilitate the formation of stable emulsions, thereby improving the bioavailability of various drugs.[38,40,41] Consequently, we hypothesized that TPGS could potentially serve as a substitute for α-tocopherol, which is essential for AS03, while concurrently formulating a stable emulsion adjuvant.

In the present study, we have formulated a novel o/w nanoemulsion adjuvant (NEA) that is devoid of α-tocopherol and incorporates squalene stabilized by TPGS. This innovative formulation is proposed as a viable alternative to AS03 for augmenting the immunogenicity of subunit vaccines. The dual role of TPGS, functioning both as an emulsifier and an immunostimulant, was effectively demonstrated. The primary physicochemical characteristics of the NEA were thoroughly characterized utilizing a range of analytical methodologies. Importantly, we evaluated the stability of the emulsion using a Turbiscan LAB stability analyzer, which employs multiple light scattering technology. Additionally, to assess the adjuvanticity of the NEA, mice were intramuscularly immunized with subunit antigens adjuvanted with NEA, specifically recombinant glycoprotein E (gE) targeting varicella-zoster virus (VZV) and highly purified hemagglutinin (HA) against seasonal influenza viruses. Our findings revealed that NEA-adjuvanted subunit vaccines induced a mixed Th1/Th2 immune response in mice and generated significantly elevated antibody titers, comparable to those elicited by AS03-adjuvanted vaccines. This study indicates that NEA represents a promising candidate for the replacement of AS03 in the formulation of effective subunit vaccines.

Materials and methods

Materials

Squalene, sodium citrate, citric acid, and polysorbate 80 (Tween 80) were procured from Hubei Gedian Humanwell Pharmaceutical Excipients Co., Ltd., located in Hubei, China. Phosphate-buffered saline (PBS) was obtained from Gibco (catalog number 10,010–031). α-Tocopherol was sourced from Sigma, while Tocopheryl Polyethylene Glycol 1000 Succinate (TPGS) was acquired from PMC Isochem. The recombinant subunit antigen of the glycoprotein E (gE) targeting the varicella-zoster virus (VZV) was produced using Chinese hamster ovary (CHO) cells at Yither Biotech Co., Ltd. in Shanghai, China. A highly purified subunit antigen of hemagglutinin (HA) was developed by Ab&B Biotech Co., Ltd. in Jiangsu, China, which included three strains of influenza virus: A/Victoria/2570/2019 (H1N1) pdm09 (A1), A/Thailand/8/2022 (H3N2) (A3), and B/Austria/1359417/2021 (B/Victoria lineage) (BV). BALB/c mice (female, aged 6–8-weeks) and C57BL/6J mice (female, aged 14–16 months) were obtained from Liaoning Changsheng Biotechnology Co. Ltd. in Liaoning, China.

Methods

Preparation of emulsion adjuvant of NEA and AS03

The NEA formulation comprises 4.3% squalene (w/v), 2% TPGS, and a 10 mm citrate buffer, prepared in accordance with previously published methodologies.[42] A specific quantity of sodium citrate (2.38 g) and citric acid (0.15 g) was dissolved in water (1 L) for injection (WFI) to create a citrate buffer with a pH of 6.5 and a concentration of 10 mm. TPGS (20 g) was fully dissolved in the aforementioned citrate buffer while being subjected to magnetic stirring at ambient temperature. Subsequently, squalene (43 g) was incorporated into the aqueous phase under continuous magnetic stirring to generate a preliminary emulsion. This preliminary emulsion underwent homogenization (13000 psi) using a microfluidizer (Microfluidics, *M*-110EH-30, USA) for four cycles, resulting in a final emulsion. In a similar manner, the AS03 adjuvant was formulated, consisting of 2.2% squalene (w/v), 2.4% α-tocopherol (w/v), 0.5% Tween 80 (w/v), and a 10 mm PBS solution. Prior to utilization, both the NEA and AS03 formulations were sterilized through 0.22 μm filtration (Millipore AS, Oslo, Norway).

Preparation of subunit vaccines

In the formulation of adjuvanted varicella-zoster virus (VZV) vaccines, the requisite volume of recombinant glycoprotein E (gE) subunit antigen solution was combined with either NEA or AS03 adjuvant, resulting in the creation of gE/NEA or gE/AS03 vaccines, each containing 100 μg of gE and 0.25 ml of adjuvant in a total volume of 0.5 ml per dose. The gE/TPGS group was composed of 100 μg of gE and 5 mg of TPGS in a single 0.5 ml dose. For comparative purposes, a control group of VZV vaccines (0.5 ml, 100 μg gE) was prepared without adjuvant by mixing the gE solution with phosphate-buffered saline (PBS).

Similarly, the adjuvanted trivalent influenza vaccines (TIV) designated as TIV/NEA or TIV/AS03 were prepared by combining the necessary volumes of hemagglutinin (HA) solution, adjuvant, and PBS to yield 15 μg of each HA and 0.25 ml of either NEA or AS03 in a 0.5 ml dose. The TIV/TPGS group for the TIV vaccines included 15 μg of each HA and 5 mg of TPGS in a 0.5 ml dose. For the TIV vaccine without adjuvant, the appropriate volumes of HA solution and PBS were mixed to achieve 15 μg of each HA in a 0.5 ml dose. All experimental vaccines were prepared under aseptic conditions, filtered through a 0.22 μm filter, and stored at 4°C until administration.

Vaccination of animal

All experimental procedures were conducted in strict compliance with the guidelines for the care and use of laboratory animals as established by the Ministry of Science and Technology of the People's Republic of China. The animal protocols received approval from the Animal Care and Use Committee at Yither Biotech Co., Ltd.

For the immunization with the VZV vaccine candidate, female mice aged 6 to 8 weeks were allocated into four distinct groups ($n = 8$ per group): gE, gE/TPGS, gE/NEA, and gE/AS03. Each mouse underwent two immunizations with either adjuvanted or unadjuvanted vaccine on days 0 and 24, receiving an intramuscular injection of 50 μl of the vaccine. Blood samples were collected three weeks post-prime and boost vaccinations. Serum was subsequently isolated via centrifugation at 3,000 rpm for 8 minutes and stored at – 80°C for subsequent antibody analysis. Following three weeks after the boost vaccination, the mice were euthanized through an intraperitoneal injection of sodium pentobarbital (80 mg/kg; Serva, Germany), and the spleen was excised for the assessment of cellular immunity.

In the case of the TIV influenza vaccine candidate, female C57BL/6J mice aged 14 to 16 months were randomly assigned to four groups ($n = 8$ per group): TIV, TIV/TPGS, TIV/NEA, and TIV/AS03. Immunization occurred on day 0, and serum samples were collected at four weeks and six weeks post-vaccination to evaluate antigen-specific binding antibodies and conduct hemagglutination inhibition (HI) assays.

Characterization on the physicochemical properties of emulsion adjuvant

The Z-average size (d, nm), size distribution (polydispersity index, PDI), and zeta potential (mV) were measured utilizing a dynamic light scattering particle sizer (Zetasizer Pro, Malvern Instruments, UK). A volume of 20 μL of each sample was diluted to 1 mL with the appropriate buffer solution and analyzed at a temperature of 25°C. The kinematic viscosity (cP) was determined using a rotary viscometer (ChemTron ALPHA, JULABO GmbH, Germany). All quantitative measurements were performed in triplicate, and the results are presented as averages.

Regarding the morphology of the emulsion droplets, 4 μL of each sample was deposited onto a grid (R2/1 Cu, 300 mesh, Quantifoil, Germany), which was subsequently immersed in liquid ethane at 4°C with a relative humidity of 100% for a duration of 3 seconds, employing a Vitrobot Mark IV System (Thermo Fisher Scientific). Imaging was conducted using a Talos F200C G2 transmission electron microscope (Thermo Fisher Scientific) operating at 200 kV. The magnification was set to 36,000 with a pixel size of 5.75 Å to facilitate the visualization of nanoparticle dimensions.

Turbiscan stability study

The Turbiscan LAB (Formulation, Toulouse, France) was employed to quantitatively and objectively assess the stability of dispersion system samples in a nondestructive manner, thereby minimizing the influence of subjective factors and elucidating the underlying causes of instability, such as aggregation or migration.[43,44] The analysis involved the transmission and scattering of light, achieved by scanning the emulsions with near-infrared light. Specifically, an 880 nm-wavelength pulsed near-infrared LED was utilized for the measurements. Parameters such as emulsion thickness and backscattering percentage were recorded. The resulting profiles provide a macroscopic representation of the emulsions at a specific time, along with insights into variations in droplet size and the occurrence of creaming or clearing phenomena. Emulsion stability was characterized by the absence of differences in backscatter diffusion, with the exception of the creaming phenomenon, which is reversible through manual agitation. Samples were scanned to a height of 43 mm, with detection occurring every minute over a 12-hour period at a controlled temperature of 25°C. The analysis focused on the variations in backscatter between NEA and AS03.

Turbiscan stability index (TSI): The comparison of the stability from formulation to formulation using only the raw Transmitted (T) or Backscattered (BS) signals can require advanced calculation. This is why the TSI has been introduced. It is crucial to classify and compare the stability of many formulations quantitatively. By using the TSI it can be done with a simple method and just one number to describe global sample stability. The TSI is a number calculated at time t by summing up all temporal and spatial variations in a considered zone:

$$\mathrm{TSI}(t) = \frac{1}{N_h} \sum_{t_i=1}^{t_{max}} \sum_{z_i=z_{min}}^{z_{max}} \mathrm{BST}(t_i, z_i) - \mathrm{BST}(t_{i-1}, z_i)$$

Equation 1. TSI algorithm equation with: Tmax the measurement point corresponding to the time t at which the TSI is calculated, Zmin and Zmax the lower and upper selected height limits respectively, Nh = (Zmin-Zmax)/Δh the number of height positions in the selected zone of the scan and BST the considered signal (BS if $T < 0.2\%$, T otherwise).

Consequently, the sample is stable when the TSI tends toward zero and unstable when the TSI is very high. Note that the TSI is equal to zero for $t = 0$.

Antigen-specific antibody analysis and hemagglutination inhibition (HI) assay of subunit TIV vaccine

The Enzyme-Linked Immunosorbent Assay (ELISA) was employed to quantify the titer of antigen-specific IgG antibodies in mouse serum. Each antigen (A1, A3 and BV) was diluted to the appropriate concentrations (A1, A3: 2.5 μg HA/mL; BV: 1.25 μg HA/mL) using a coating buffer, followed by the overnight coating of 96-well plates with the respective antigens at 4°C. The serum samples were serially diluted in a 3-fold manner, commencing at a dilution of 1:100, and subsequently added to plates coated with 5% milk, incubating at 37°C for 1 hour. Following a wash with PBS, the plates were incubated with HRP-conjugated goat anti-mouse IgG (Beyotime Biotechnology, A0216, 1:250) at 37°C for 1 hour. The colorimetric reaction was initiated by the addition of 100 μL of TMB (Beyotime Biotechnology, P0209) for 10 minutes, after which 50 μL of an ELISA terminating solution (Beyotime Biotechnology, P0215) was added to halt the reaction. Absorbance was measured at 450 nm using an ELISA plate reader (Thermo Fisher Scientific). The mean optical density (OD) of serum from naive mice was multiplied by 2.1 to establish the positive cutoff point, with a lower limit for antibody titer detection set at 100.

To eliminate nonspecific inhibitors from the mouse serum, an overnight treatment with receptor-destroying enzymes (Merck, C8772) was conducted. The serum was then subjected to a 2-fold serial dilution in 96-well plates, starting at a dilution of 1:10, followed by the addition of 4 hA units of each antigen (A1, A3, BV). Control wells received saline only. After a 60-minute incubation at room temperature, 25 μL of a 1% solution of chicken red blood cells (for A1, BV) or guinea pig red blood cells (for A3) was introduced into the mixture, which was then incubated for an additional 30 minutes (for A1,BV) or 60 minutes (for A3) prior to assessing hemagglutination. The hemagglutination inhibition (HI) titer was recorded as the reciprocal of the highest serum dilution at which hemagglutination was inhibited, with a lower limit for antibody titer detection established at 10.

ELISA and enzyme-linked immunospot (ELISPOT) assay of subunit VZV vaccine

A recombinant gE protein at a concentration of 2 μg/ml was utilized to precoat 96-well microtiter plates overnight at 4°C. Following this, the plates were washed three times with a solution of 0.05% (vol/vol) polysorbate 20 in phosphate-buffered saline (PBS) and subsequently blocked with a 5% (w/v) skim milk solution in PBS for one hour. Two-fold serial dilutions of mouse serum samples were then incubated in the plates containing 1% skim milk for one hour. Detection of antibodies was achieved using goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) at a dilution of 1:10,000 (Bio-Rad, Hercules, CA, USA). Five minutes post-addition of the substrate 3,3,5,5-tetramethylbenzidine (TMB; BD, San Diego, CA, USA), the reaction was terminated by the addition of 1 M sulfuric acid. The optical density at 450 nm was measured using a spectrophotometer (BioTek Instruments, Inc.,

Winooski, VT, USA). IgG titers were determined as the maximum dilution factor that yielded cutoff signals exceeding an optical density of 0.15. Based on this criterion, serum antibody titers exhibiting an optical density of less than 0.10 at a dilution of 1:2000 were assigned a value of 100 for the purposes of statistical analysis. The titers were reported as log10 mean (±SEM) endpoint titers, utilizing a cutoff of 2.1 times the mean of the blank wells. Antibody titers were calculated through linear interpolation and expressed as endpoint titers.

To evaluate the production of IL-2 and IFN-γ, an enzyme-linked immunospot (ELISPOT) assay was conducted using an ELISPOT assay kit (BD) in accordance with the manufacturer's guidelines. In brief, splenocytes harvested from immunized mice were plated in 96-well plates, with final concentrations of $5 \times 10\text{^}5$ cells/well and $2 \times 10\text{^}5$ cells/well, respectively. The gE protein was employed at a final concentration of 20 μg/ml to elicit gE-specific T cell responses over a 16-hour period, while equivalent volumes of medium or PMA + ionomycin served as negative and positive controls, respectively. Following the removal of cell supernatants, spots were developed using an ALL-IN-ONE mouse ELISPOT accessory kit and quantified using an ELISPOT reading system (Autoimmun Diagnostika GmbH, Strassberg, Germany).

Assess the safety of NEA in mice

In order to evaluate the safety of the NEA in a murine model, female BALB/C mice, aged 6 to 8 weeks and weighing between 15 to 20 grams, were randomly assigned to three distinct groups (5 mice per group): the negative control group (Group 1) received normal saline, the experimental group (Group 2) was administered the NEA, and the control group (Group 3) received AS03. All mice were maintained in a specific pathogen-free environment. The normal saline or adjuvants of NEA and AS03 were delivered via intramuscular injection in the hind limb, with two immunizations occurring two weeks apart (day 0 and day 14), each at a dosage of 50 μL. Observations regarding allergic reactions, responses at the injection site, such as redness, induration, hair loss and other relevant conditions, were conducted on the zero,1st, 3rd, 5th, 7th, 9th, 14th, 15th, 17th, 19th, and 21st days following the initial administration. Body temperature was measured using an infrared thermometer, and body weight was assessed with an electronic balance.

Statistical analysis

For the data animal immunogenicity, statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, SanDiego, CA, USA). Differences between the two groups were analyzed with unpaired t-tests. Significance level was marked in figures and annotated in the figure legends.

Results and discussion

Physicochemical properties of emulsion adjuvant

The effective development and production of high-quality emulsion-based products are contingent upon a comprehensive understanding of their physicochemical properties and stability. The establishment of emulsion systems and the preservation of this state for the requisite duration of the desired processes constitute a fundamental operation in the manufacturing of emulsion aids.[45,46] Achieving such a state necessitates the precise selection of an emulsifier, which is critical for reducing the interfacial tension between the aqueous and oil phases, thereby mitigating the occurrence of undesirable phenomena such as creaming, sedimentation, and phase inversion. Table 1 presents a summary of the physicochemical properties of NEA and AS03.

The NEA formulation, which contains 4.3% (w/v) squalene oil stabilized by 2% (w/v) TPGS emulsifier in a 10 mm citrate buffer, was prepared using high-pressure microfluidic homogenization.[42] In comparison, a standard adult human vaccine dose, referred to as AS03A or simply AS03 in this article, comprises 0.97% polysorbate 80 (Tween 80), 2.2% squalene, and 2.4% α-tocopherol.[18] The size and distribution of emulsion droplets are critical factors influencing their dispersion stability. As indicated in Table 1 and Figure 1a, the emulsion droplet size of NEA is measured at 160.1 ± 1.4 nm, exhibiting an exceptionally narrow size distribution (PDI = 0.11 ± 0.01).

The emulsion droplet size of NEA is comparable to that of AS03 (Figure 1c, size = 153.0 ± 1.2 nm, PDI = 0.14 ± 0.01). Zeta potential measurements revealed that both NEA and AS03 emulsions possess a negative charge at neutral pH, with zeta potential values of approximately −23 mV. The morphology of the emulsions was analyzed using transmission electron microscopy (TEM). As illustrated in Figures 1b,d, both NEA and AS03 exhibit a distinct spherical morphology, with a few particles displaying irregular edges due to uneven shrinkage during sample preparation. Notably, the remarkably low kinematic viscosity (cP) value of NEA (1.91 ± 0.02), which is close to that of water, suggests the presence of an o/w emulsion system, thereby enhancing its syringeability.

Turbiscan stability of emulsion adjuvant

TPGS is a widely utilized emulsifier across the food, pharmaceutical, and cosmetic industries. Our previous research[42] examined the influence of TPGS concentration on the droplet size and PDI of squalene-based emulsions. We determined that at an optimal TPGS concentration of 2%, the NEA demonstrated remarkable storage stability at temperature of 2–8 °C for over one year. To further assess the potential of NEA as a substitute for AS03, we evaluated the emulsion's stability

Table 1. Summary of physicochemical properties of NEA and AS03.

Item	NEA	AS03
Composition	2% TPGS 4.3% Squalene 10.0 mm Citrate buffer	0.97%Tween80 2.2% Squalene 2.4% α-tocopherol 10.0 mm PBS buffer
pH	6.5 ± 0.1	6.5 ± 0.1
Particle size (nm)	160.1 ± 1.4	153.0 ± 1.2
PDI	0.11 ± 0.01	0.14 ± 0.01
Zeta potential (mV)	−23.5 ± 1.3	−22.7 ± 2.1
Kinematic viscosity (cP)	1.91 ± 0.02	2.13 ± 0.89

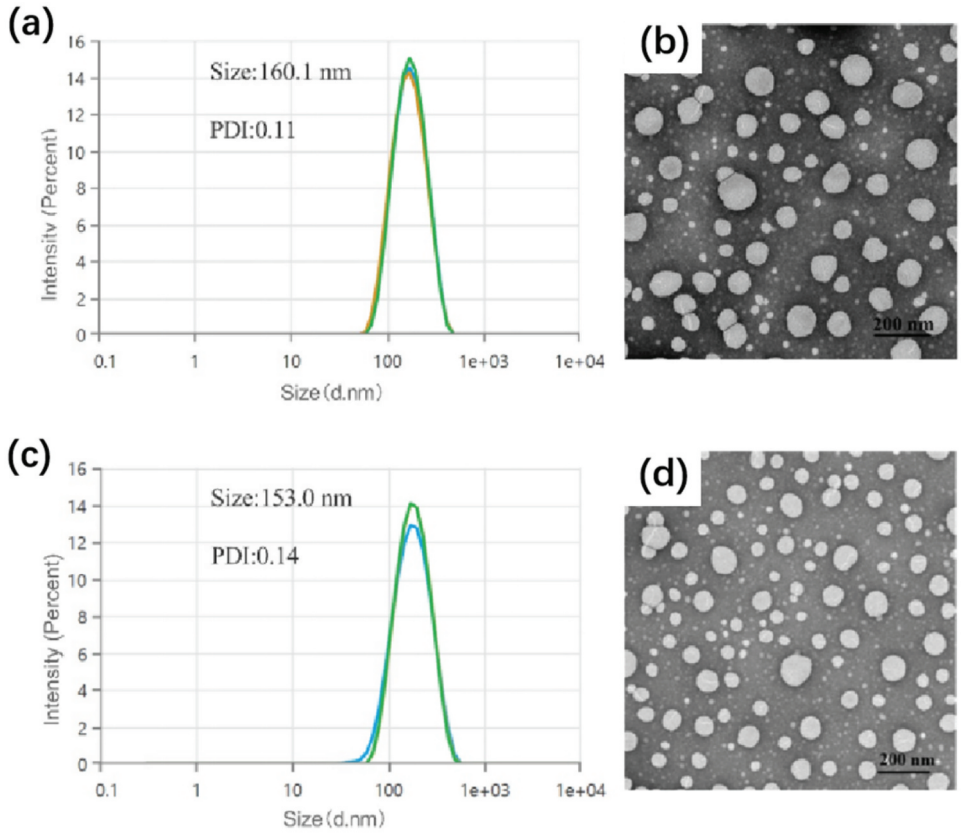

Figure 1. Emulsion droplet size distribution (a: NEA, c: AS03) and morphology (b: NEA, d: AS03) determined by DLS and cryo-TEM, respectively.

using a Turbiscan LAB stability analyzer (Formulaction, France), which employs multiple light scattering technology.

Figure 2 illustrates the variations in backscattering intensity (BS) in relation to the length of the measuring cell and the duration of the scanning process. The horizontal axis represents the height of the sample from the bottom to the top, while the vertical axis indicates the changes in the intensity of the backscattered light. The spectrum features different colored curves that correspond to various time points during the measurement. The data collection spans a 12-hour time frame, with blue indicating the earliest time and red denoting the latest time.

The backscatter signals from AS03 (Figure 2a) and NEA (Figure 2b) exhibit a uniform distribution across height, commencing at minimal intensities and experiencing a slight increase at a height of 37 mm within the bottle. Up to the measuring cell height of 43 mm, a modest increase in backscatter (BS) intensity was recorded, reaching 15.5% for NEA and 11% for AS03, respectively. This observation suggests a positive correlation between backscattered light intensity and concentration, indicating a decrease in concentration at the bottom of the sample and an increase at the top. Consequently, both NEA and AS03 exemplify the uplift process devoid of flocculation, agglomeration, and delamination of the emulsion.[43,47]

The peak thickness at a height of 43 mm in the up-floating section was determined using Turbiscan software. A comparison of the peak thickness illustrated in Figure 2c reveals that the peak thickness began to rise after 0.5 hours, reaching a maximum of 1 mm after six hours, and remaining below 1.4 mm after 12 hours for both AS03 and NEA (Table 2). The peak height and layered thickness at the top of AS03 were marginally greater than those of NEA, suggesting a higher concentration of emulsion droplets at the top of AS03. Conversely, the peak height and layered thickness at the bottom of NEA were slightly larger than those of AS03 (Table 2), indicating a greater concentration of layered emulsion droplets at the bottom of NEA.[47]

The Turbiscan Stability Index (TSI), which ranges from 0 to 100, reflects the variability in multiple scans of light intensity received by the emulsions, encompassing changes in agglomeration and delamination stability of the sample. According to Equation 1, the sample is stable when the TSI tends toward zero and unstable when the TSI is very high. A higher TSI value indicates poorer emulsion stability.[44,48] As presented in Table 2, both NEA and AS03 exhibited ultra-low TSI values of 0.34, signifying that both emulsions possess commendable stability.

Adjuvanticity of NEA in varicella-zoster viruses (VZV) vaccine

In elderly or immunocompromised populations, VZV cannot be effectively managed due to insufficient cellular immunity, resulting in the manifestation of herpes zoster.[49] The current study investigates the immunogenicity of a subunit recombinant glycoprotein E (gE) in conjunction with various adjuvants in an animal model. We evaluated the impact of different

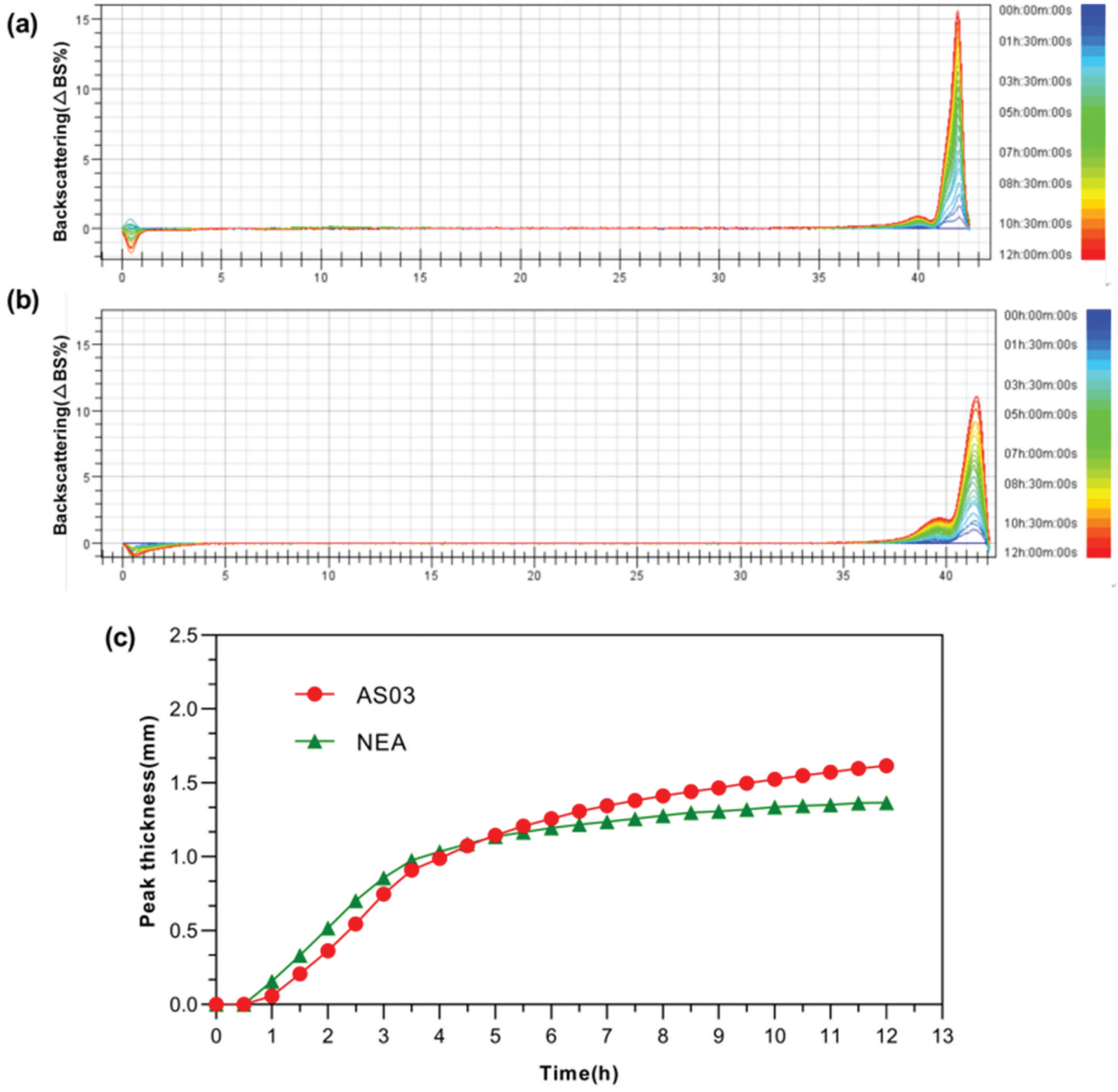

Figure 2. Backscattering profiles of AS03 (a) and NEA (b) subjected to continuous accelerated aging for 12 h at 25°C; (c) peak thickness curves of AS03 and NEA emulsions after 12 h of continuous accelerated aging.

Table 2. Summary of Turbiscan stability studied at accelerated conditions (25°C).

Samples	Turbiscan stability index (TSI)	Topographic height (%)	Top layering thickness (mm)	Bottom peak height (%)	Bottom layering thickness (mm)
NEA	0.34	13.41	1.33	−0.48	4.50
AS03	0.34	15.60	1.37	−1.32	0.89

adjuvant formulations, specifically gE, gE/TPGS, gE/NEA, and gE/AS03, by measuring gE-specific antibody and T cell responses three weeks following the initial and booster vaccinations. The gE-specific serum immunoglobulin G (IgG) responses were quantified using enzyme-linked immunosorbent assay (ELISA) (Figure 3), while gE-specific T cell responses, characterized by interleukin-2 (IL-2) and interferon-gamma (IFN-γ) production, were assessed through ELISpot assays (Figure 4) following ex vivo stimulation of splenocytes with the gE protein.

As illustrated in Figure 3a, three weeks following primary immunization, the gE/TPGS formulation elicited significantly elevated gE-specific IgG ELISA titers compared to the gE alone group ($p < .05$), thereby demonstrating the capacity of TPGS to

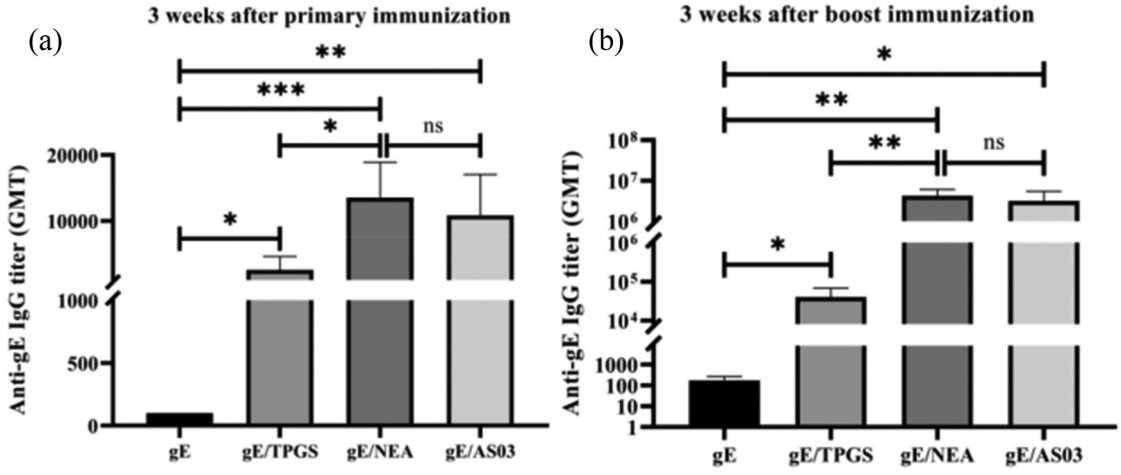

Figure 3. Anti-gE IgG titers of gE, gE/TPGS, gE/NEA and gE/AS03 at 3 weeks after primary (a) and boost (b) vaccination determined by Elisa. ns: $p \geq .05$ between the two groups, *$p < .05$ between the two groups, **$p < .01$ between the two groups, ***$p < .001$ between the two groups.

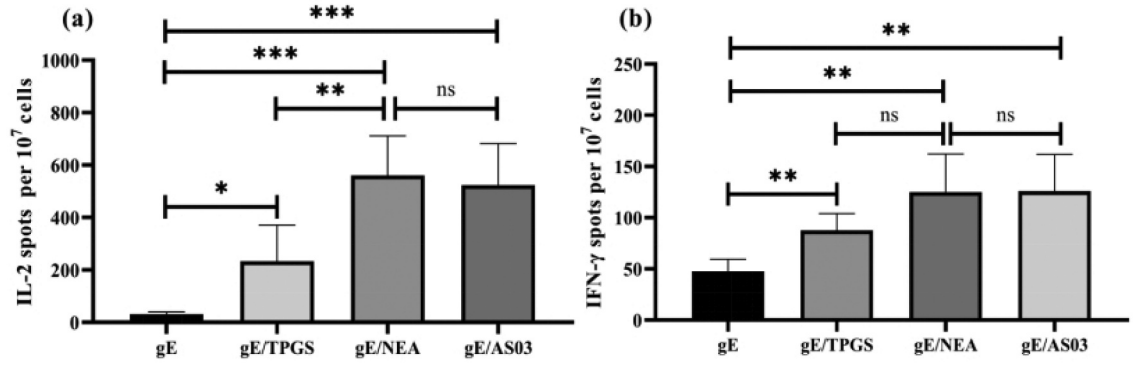

Figure 4. gE-specific IL-2 (a) and ifn-γ-secreting T cells (b) of gE, gE/TPGS, gE/NEA and gE/AS03 at 3 weeks after boost vaccination determined by ELISpot. ns: $p \geq .05$ between the two groups, *$p < .05$ between the two groups, **$p < .01$ between the two groups, ***$p < .001$ between the two groups.

enhance the immune response in murine models. Upon the incorporation of TPGS into the emulsion system, specifically NEA, a marked increase in gE-specific total IgG was observed in the NEA/gE group relative to the gE/TPGS group ($p < .001$). Both the gE/TPGS and gE/NEA groups exhibited increased total IgG titers three weeks post-boost vaccination, with a statistically significant difference maintained between the two groups ($p < .01$), further underscoring the necessity of TPGS in the formulation of emulsions. When AS03 was utilized as an adjuvant, the gE/AS03 group demonstrated stronger gE-specific immune responses compared to the gE group ($p < .01$ and $p < .05$ following the primary and boost vaccinations, respectively). No statistically significant difference ($p \geq .05$) was noted between the gE/NEA and gE/AS03 groups, indicating an equivalent adjuvant effect between NEA and AS03.

TH1 cells, distinct from CD4+ T cells, primarily secrete cytokines such as IFN-γ, IL-2, and TNF-β, which are integral to the cellular immune response and play a crucial role in combating infections caused by intracellular pathogens. We evaluated gE-specific T cell responses through intracellular cytokine staining (ICS) for IFN-γ and IL-2 following ex vivo stimulation of splenocytes. In the absence of an adjuvant, the gE group exhibited minimal production of IL-2 (Figure 4a) and IFN-γ (Figure 4b), indicating that immunization with the gE antigen alone primarily induces TH2 rather than TH1 responses. In contrast, the inclusion of TPGS, NEA, or AS03 as adjuvants resulted in elevated levels of IL-2 and IFN-γ across all groups compared to the gE group alone. Notably, the gE/TPGS group demonstrated the most significant increases in gE-specific IL-2 ($p < .05$) and IFN-γ secretion ($p < .01$) relative to the gE group. Upon switching the adjuvant to NEA, a significant difference in IL-2 levels ($p < .001$) was observed between the gE/NEA and gE/TPGS groups, while no significant difference was noted for IFN-γ ($p > .05$). Moreover, there was no statistical difference in IL-2 ($p \geq .05$) and IFN-γ($p \geq .05$) levels between gE/NEA and gE/AS03 groups. Collectively, these findings indicate that the gE/NEA formulation elicits gE-specific immune responses that are comparable to those induced by gE/AS03.

Adjuvanticity of NEA in seasonal trivalent influenza (TIV) vaccine

The incorporation of adjuvants in influenza vaccines has been demonstrated to enhance antibody responses in older populations, thereby improving protection against severe illness and decreasing both morbidity and mortality rates.[21,50] During the

A/H1N1pdm09 pandemic, two AS03-adjuvanted split-virion vaccines, Pandemrix and Arepanrix H1N1,[51] were developed and licensed to target the A/California/7/2009 h1N1 strain. These adjuvanted vaccines exhibit greater immunogenicity compared to their unadjuvanted counterparts, induce cross-reactive immunity against sub-clade variants, and allow for antigen sparing. Consequently, we investigated the efficacy of NEA as an adjuvant for the seasonal trivalent influenza vaccine (TIV) in a murine model. Mice were immunized intramuscularly with subunit TIV, TIV/TPGS, TIV/NEA, or TIV/AS03 on days 0 and 14. Serum samples were collected from all immunized mice at 4 and 6 weeks post-vaccination, and the total IgG antibody responses are illustrated in Figure 5.

The TIV/TPGS group did not demonstrate a significantly enhanced immunogenicity compared to TIV alone across all three HA antigens, as indicated by comparable IgG titers ($p \geq .05$). Notably, at 4 weeks post-vaccination, significant differences in IgG titers were observed between the TIV/NEA and TIV groups for H1N1 ($p < .01$), H3N2 ($p < .05$), and BV ($p < .05$). A similar pattern was noted at 6 weeks post-vaccination, with significant differences for H1N1 ($p < .01$), H3N2 ($p < .05$), and BV ($p < .05$). Remarkably, serum IgG

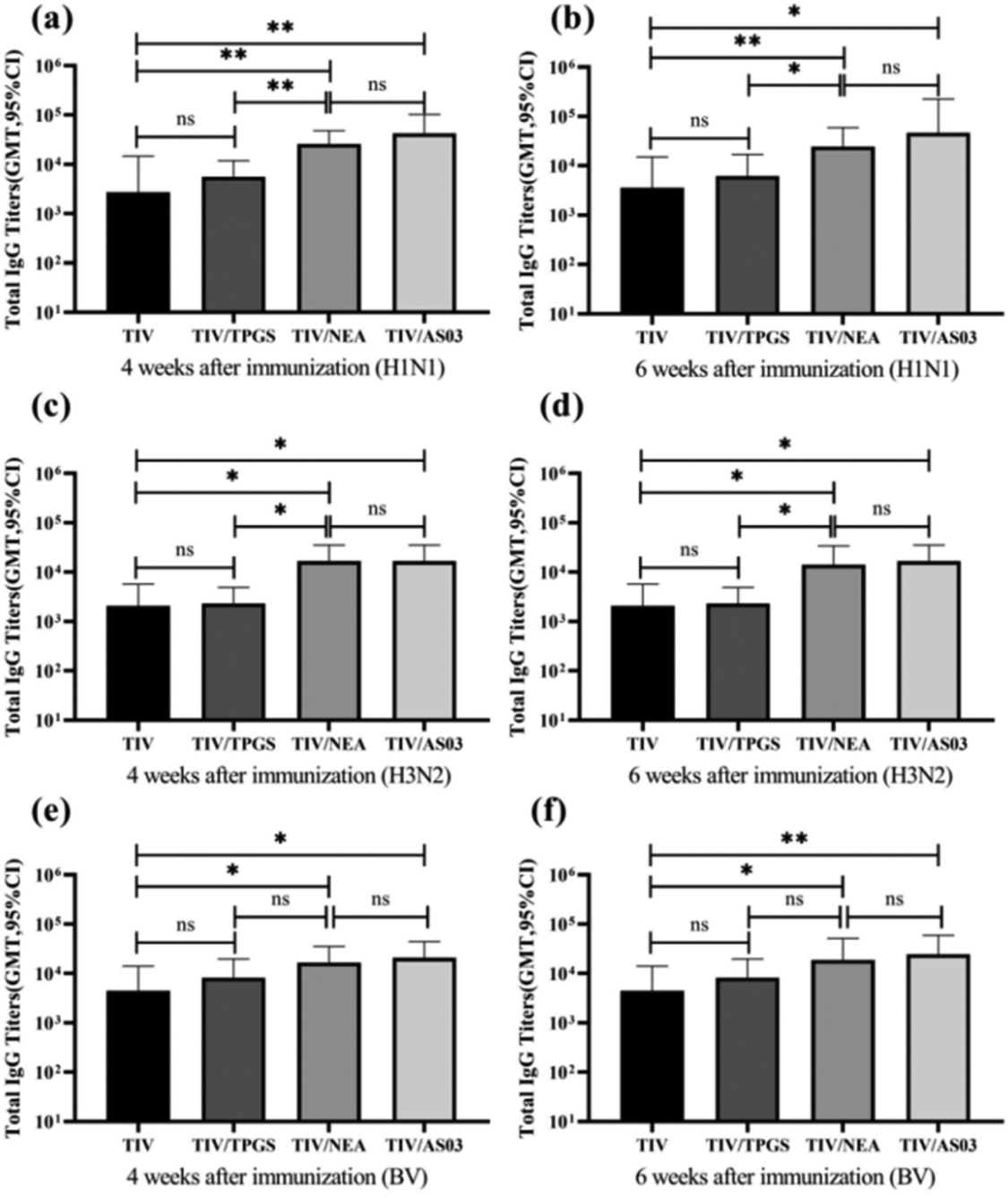

Figure 5. Mice vaccination with trivalent influenza vaccine (TIV) alone or adjuvanted TIV (TIV/TPGS, TIV/NEA and TIV/AS03). Anti-H1N1(d), anti-H3N2(e) and anti-bv (f) IgG antibody titers at 4- (a, c and e) and 6- (b, d and f) weeks determined by ELISA. ns: $p \geq .05$ between the two groups, *$p < .05$ between the two groups, **$p < .01$ between the two groups, ***$p < .001$ between the two groups.

levels against H1N1, H3N2, and BV in the TIV/NEA group were comparable to those in the TIV/AS03 group at both 4 and 6 weeks post-vaccination ($p \geq .05$).

The hemagglutination inhibition (HI) assay was employed to assess the anti-HA antibody response, with Figure 6 summarizing the geometric mean titers (GMT) of HI titers at 4 and 6 weeks post-vaccination for the various vaccine formulations.

At 4 weeks, a statistically significant difference was noted between the TIV/TPGS and TIV groups for H1N1 (Figure 6a, $p < .05$), while no significant differences were observed for H3N2 and BV (Figure 6c,e, $p \geq .05$). The TIV/NEA and TIV/AS03 groups elicited significantly higher HI titers against H1N1, H3N2, and BV compared to TIV alone, with no significant differences between TIV/NEA and TIV/AS03. Interestingly, the TIV/NEA group exhibited significantly higher HI titers than TIV/TPGS for H1N1 and H3N2, although equivalent HI titers were observed for BV. After 6 weeks of vaccination (Figures 6b,d,f), TPGS did not elicit higher HI titers, while NEA and AS03 maintained their adjuvanticity. No statistically significant differences were found between TIV/NEA and TIV/AS03 for any of the three HA types. These findings suggest that the experimental vaccine TIV/NEA induces a more robust immune response than the TIV vaccine when administered intramuscularly, potentially offering effective protection against influenza virus infection.

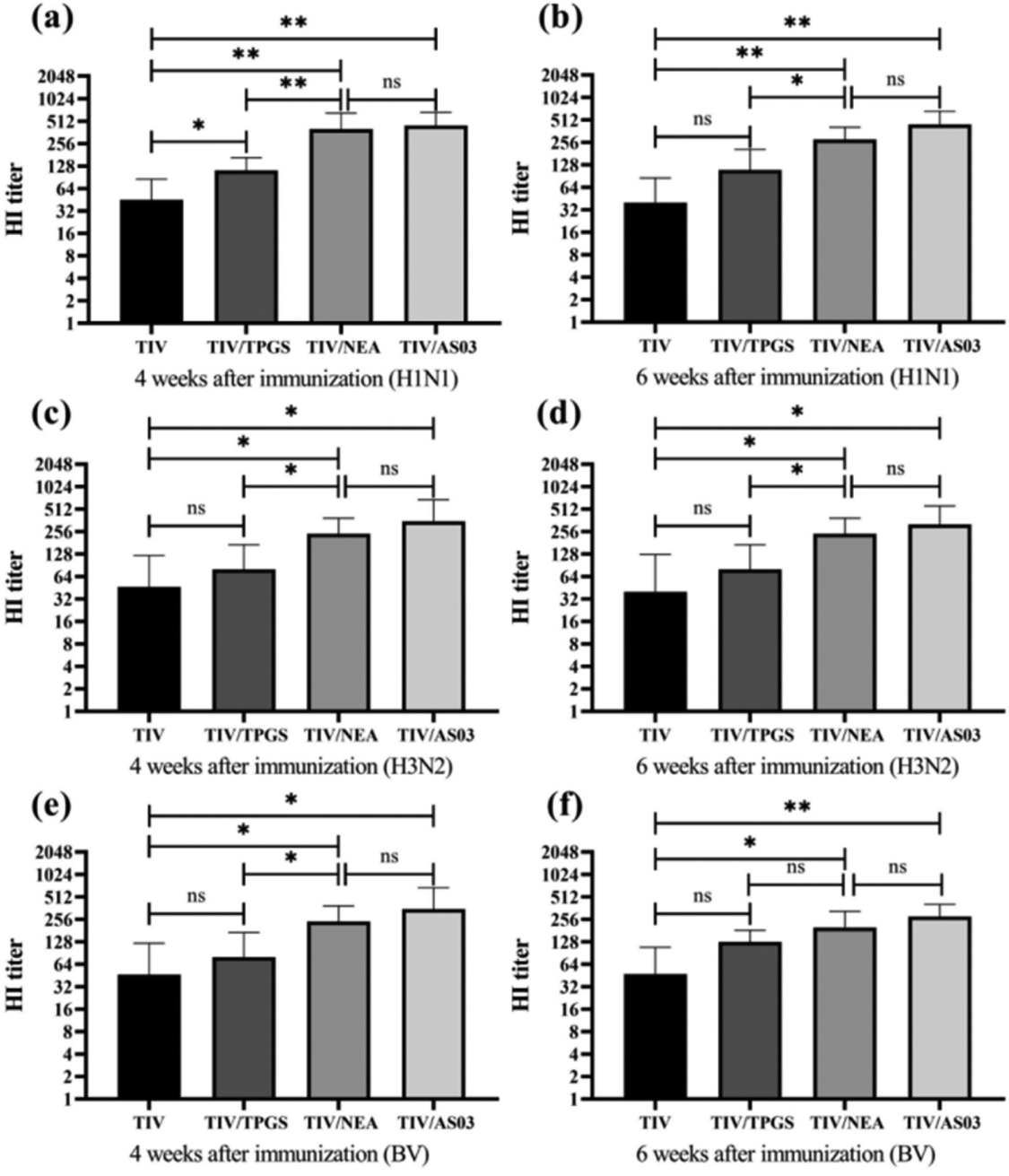

Figure 6. Immunization of trivalent influenza vaccine (TIV) or adjuvanted TIV (TIV/TPGS, TIV/NEA and TIV/AS03). Anti-H1N1 (a,b), anti-H3N2 (c,d) and anti-bv (e,f) HI titers at 4- (a, c and e) and 6- (b, d and f) weeks determined by HI assay. ns: $p \geq .05$ between the two groups, *$p <$. 05 between the two groups, **$p < .01$ between the two groups, ***$p <$. 001 between the two groups.

The safety evaluation of NEA in mice

To evaluate the safety profile of NEA and to provide foundational evidence for future clinical trials, NEA was twice administrated to young BALB/C mice and assessed weight lose, mortality and body temperature, observations regarding allergic reactions response at the injection site, such as redness, induration and hair loss were also recorded during twice administration.

Throughout the course of the experiment, no allergic reactions or mortality were recorded among the mice, and instances of alopecia were not significant. When compared to the negative control group, the NEA group exhibited a slight degree of erythema, swelling, and induration at the injection site, which was consistent with the observations noted in the AS03 control group. The body temperatures of all three groups remained within the defined range of 36.2 ± 0.1°C to 36.4 ± 0.1°C, with no significant deviations noted. The changes in body weight are depicted in Figure 7. Mice in the saline group demonstrated a gradual increase in body weight, rising from 17.7 g to 19.1 g. Following each administration of AS03, a decrease in body weight was observed in the mice, although this effect was less pronounced in the NEA group. Both the NEA and AS03 groups exhibited an overall upward trend in body weight but the increase was relatively lower compared to that of the normal saline group. These preliminary findings suggest that NEA does not exhibit significant toxic effects.

TPGS needs to be formulated to emulsion for enhancing its adjuvanticity

Metabolizable o/w emulsions have been extensively utilized as effective vaccine adjuvants. Among these, squalene is often regarded as the preferred metabolizable oil; however, a variety of emulsifier compositions are employed in both human and veterinary products.[4,19,52] The selection of emulsifiers is primarily determined by their capacity to stabilize emulsions and/or their biological activity. For instance, the MF59 adjuvant incorporates Tween 80 and sorbitan trioleate (Span 85), whereas AS03 exclusively utilizes Tween 80.[18] The schematic representation of the emulsion droplet for AS03 is illustrated in Figure 8. The adjuvant properties of AS03 are attributed to both the o/w emulsion system and the presence of α-tocopherol, while neither squalene nor Tween 80 exhibits immune-stimulating functions. Research has indicated that larger emulsion droplets (approximately 160 nm) enhance the recruitment of immune cells to the injection site, leading to improved antigen uptake, expedited translocation to draining lymph nodes, and enhanced cellular and humoral immune responses.[11] Here, we formulated a NEA using squalene as the oil phase and TPGS as the emulsifier. The resulting NEA demonstrated a droplet size comparable to that of AS03 (approximately 160 nm) and exhibited similar physicochemical properties, thereby facilitating the investigation of TPGS as a potential substitute for AS03.

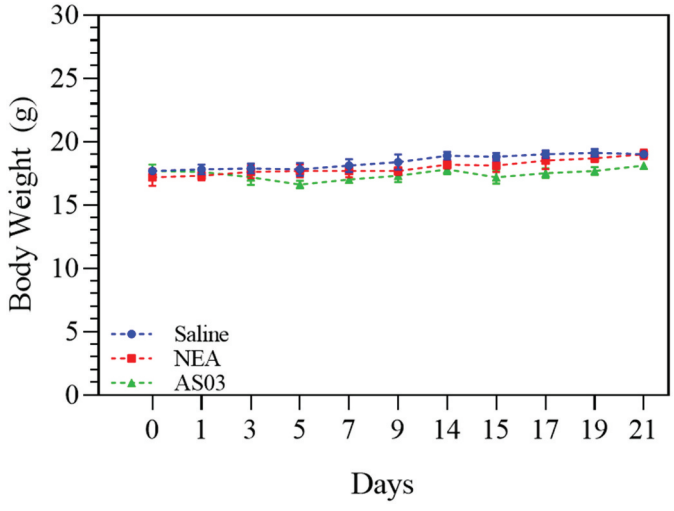

Figure 7. Variation in body weight as a function of days (twice administration at day 0 and day 14).

Subunit vaccines adjuvanted with TPGS elicited a more robust immunogenic response, particularly with respect to the model antigen gE protein, compared to gE administered alone, indicating the adjuvanticity of TPGS. The chemical structure of TPGS, as depicted in Figure 8, reveals the presence of tocopheryl succinate ester groups with polyethylene glycol (PEG) chains. The structural similarity of the α-tocopheryl succinate ester to α-tocopherol suggests that TPGS may function analogously to α-tocopherol as an immunomodulator. The tocopheryl structure may influence cytokine production, as well as the early migration of eosinophils and neutrophils to the draining lymph nodes and the antigen-loading process in monocytes.[12,13,20] PEG is a biocompatible polymer frequently conjugated to therapeutic agents to enhance their bioavailability and therapeutic effectiveness. Although it is commonly assumed that PEG possesses low immunogenicity, a substantial body of research and clinical evidence has revealed the presence of moderate to significant anti-PEG antibodies in individuals who have not previously been exposed to PEGylated medications.[53,54] As a result, it is currently difficult to determine whether PEG, either independently or in combination with the α-tocopheryl structure, plays a role in exhibiting adjuvant properties for TPGS. While TPGS alone was effective for the model antigen gE protein, it did not induce a robust antibody response to the model antigen HA protein. Notably, NEA exhibited superior adjuvanticity compared to both gE and HA proteins administered alone, as well as compared to antigens adjuvanted with TPGS. The administration of a vaccine containing the emulsion adjuvant facilitates the recruitment of a significant number of immune cells to the injection site, thereby enhancing antigen absorption and presentation by these immune cells.[10–14] Consequently, it is imperative for TPGS to form an emulsion system to achieve the desired adjuvanticity.

NEA, in contrast to AS03, does not incorporate α-tocopherol; rather, it utilizes TPGS, a derivative of α-tocopherol, effectively eliciting a strong immunogenic response to subunit vaccines in murine models. This derivative is commonly found in dietary supplements and skincare formulations, exhibiting greater stability and a reduced susceptibility to oxidation compared to α-tocopherol. Consequently, TPGS facilitates the formation of a more stable emulsion than

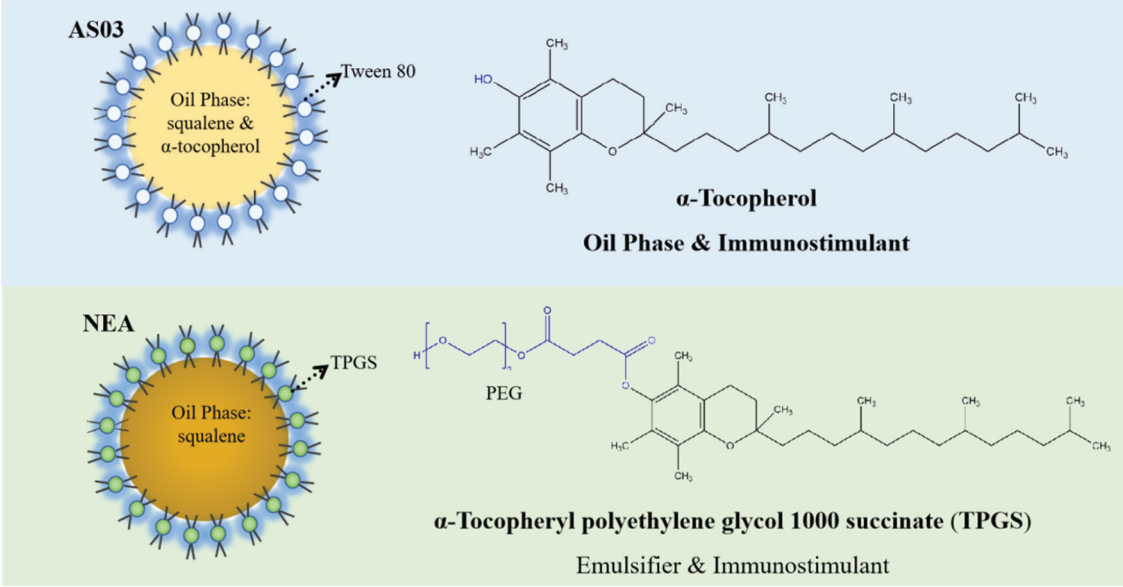

Figure 8. Schematic of emulsion droplet of AS03 and NEA.

AS03. Furthermore, TPGS is recognized as an FDA-approved pharmaceutical excipient and is more cost-effective than α-tocopherol, which enhances its scalability. Given that TPGS possesses adjuvant properties, NEA based on TPGS may have the potential to optimize immune responses in the body, thereby addressing emerging infectious diseases and combating the challenge of drug resistance. However, the extensive clinical application of NEA faces several significant challenges. Long-term monitoring is essential to evaluate potential adverse effects, and the safety of these interventions in specific populations, such as children and pregnant women, requires further validation. Variability in immune responses across different demographic groups necessitates the optimization of adjuvants to enhance their efficacy. Additionally, it is crucial to ensure the longevity of immune protection while minimizing the frequency of vaccinations. Other considerations include the need for regulatory approval, financial implications, public acceptance, the logistics associated with cold chain distribution, and the capacity to address emerging infectious diseases. Overcoming these obstacles will require collaborative efforts and ongoing innovation.

Conclusion

This study presents a novel NEA that utilizes the dual properties of TPGS as both an emulsifier and an immunostimulant. Although the components of NEA and AS03 differ, their physicochemical properties exhibit significant similarities, including particle size/PDI, morphology, pH, zeta potential and viscosity. The NEA displayed a typical uplift process, characterized by the absence of flocculation, agglomeration, and delamination, and demonstrated commendable stability akin to AS03, as evidenced by a TSI of less than 1. Immunization with the gE antigen in combination with NEA resulted in markedly enhanced antigen-specific humoral and cellular immune responses compared to gE administered alone, achieving levels comparable to those induced by gE/AS03 in mice. Furthermore, the incorporation of NEA into TIV significantly increased anti-influenza antibody levels in mice, exceeding those observed in non-adjuvanted TIV and matching the responses elicited by AS03-adjuvanted formulations. The presence of TPGS and its emulsion formulation are critical factors contributing to the robust adjuvanticity of NEA. In terms of scalability, NEA is comparable to AS03, as it can be readily produced through homogenization, thus presenting no significant scalability challenges. Since TPGS is more cost-effective than α-tocopherol, it is a more efficient choice for NEA production compared to AS03. The reported NEA has the potential to serve as an alternative to the AS03 adjuvant, thereby improving vaccine accessibility, fostering technological innovation, and enhancing global capacity to respond to infectious diseases. Nonetheless, its practical application remains limited due to insufficient long-term stability data and a comprehensive non-clinical toxicological assessment. Subsequent investigations will be undertaken to support the progression toward clinical trials.

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Author contributions

Quanyi Yin: Conceptualization, methodology, validation, writing – original draft preparation, writing – review and editing; Shuoyao Song: Data curation, software, formal analysis, investigation; Zhilei Liu: Resources, supervision, review. All authors have read and agreed to the published version of the manuscript.

Institutional review board statement

All experiments were performed strictly in accordance with the guidelines of care and use of laboratory animals by the Ministry of Science and Technology of the People's Republic of China. The animal study protocol was approved by the Animal Care and Use Committee of Yither Biotech Co., Ltd.

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