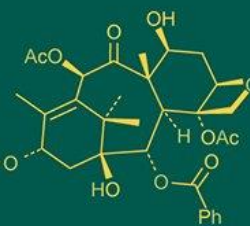
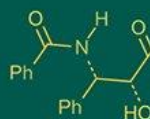


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Formulation and characterization of a nanoemulsion-based vaccine adjuvant

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Abstract

Nanoemulsions are colloidal dispersions consisting of oil and water phases stabilized by surfactants. Owing to their nanoscale dimensions, nanoemulsions exhibit high kinetic stability, large interfacial surface area, enhanced dispersibility, and optical transparency resulting from minimal light scattering. These unique physicochemical properties have led to their widespread application in pharmaceutical, cosmetic, and food industries as efficient delivery systems for bioactive compounds. In recent years, nanoemulsion-based systems have gained considerable attention as vaccine adjuvants due to their ability to enhance antigen uptake, improve immune stimulation, and facilitate controlled antigen release. Compared with conventional emulsion-based adjuvants, nanoemulsions offer improved stability, biocompatibility, and safety profiles, making them attractive candidates for both human and veterinary vaccine formulations. The advancement of recombinant DNA technology has accelerated the development of highly purified recombinant antigens. Although these antigens exhibit excellent safety characteristics, they often possess limited intrinsic immunogenicity due to their rapid degradability and therefore require suitable adjuvants to induce robust and long-lasting immune responses. Several adjuvant systems, including mineral oils, aluminium salts, bacterial derivatives, and live attenuated microorganisms, have been employed to enhance vaccine efficacy. However, concerns regarding reactogenicity, stability, and immunological performance continue to drive the search for novel adjuvant platforms. Mineral oil-based nanoemulsions represent a promising alternative owing to their physicochemical stability, ease of formulation, and potential immunostimulatory properties. Nevertheless, comprehensive characterization of such formulations is essential before their incorporation into vaccine delivery systems. Therefore, the present study was undertaken to develop a novel mineral oil-based nanoemulsion using a sonication approach and to evaluate its physicochemical characteristics. The formulated nanoemulsion was characterized for particle size distribution, zeta potential, surface morphology, and thermodynamic stability. In addition, its preliminary safety profile was assessed in guinea pigs to determine its suitability as a potential vaccine adjuvant.

Keywords: Nanoemulsion, thermodynamic stability, zeta potential, polydispersibility, safety

1. Introduction

Nanoemulsions are colloidal dispersions consisting of two immiscible liquids stabilized by surfactants. Nanoemulsions exhibit unique physicochemical properties, including high surface area, optical transparency, enhanced dispersibility, and remarkable kinetic stability. Their long-term physical stability against flocculation, coalescence, and sedimentation makes them highly attractive for pharmaceutical and biomedical applications. This exceptional stability is largely attributed to steric stabilization imparted by non-ionic surfactants and polymers, resulting in systems that are often described as approaching thermodynamic stability (Tadros *et al.*, 2004)^[13]. Over the past two decades, nanoemulsions have emerged as versatile delivery platforms in pharmaceutical, cosmetic, food, and biotechnology industries. Compared with conventional emulsions and microemulsions, nanoemulsions possess several advantages, including enhanced physical stability, lower surfactant requirements, improved drug solubilization, and superior bioavailability of poorly water-soluble compounds. The extremely small droplet size minimizes gravitational separation and reduces the tendency for droplet aggregation and coalescence, thereby maintaining formulation homogeneity during storage. Furthermore, the large interfacial surface area facilitates rapid absorption and penetration of encapsulated compounds across biological barriers, making nanoemulsions particularly suitable for targeted and controlled delivery applications (Tharwat *et al.*, 2003)^[14].

Nanoemulsion-based formulations can be administered through multiple routes, including parenteral, oral, topical, nasal, pulmonary, and ocular pathways. Nanoemulsions have gained considerable attention as vaccine adjuvants owing to their ability to enhance antigen uptake, promote antigen presentation, and stimulate both humoral and cellular immune responses. Their nanoscale dimensions facilitate efficient interaction with antigen-presenting cells, resulting in improved immunogenicity and prolonged immune stimulation compared with conventional adjuvant systems.

Several studies have demonstrated the potential of nanoemulsion-based adjuvants in vaccine development. The oil-in-water nanoemulsion adjuvant W805EC has been shown to significantly enhance immune responses against influenza virus antigens, inducing both Th1- and Th17-mediated immunity while maintaining a favorable safety profile (Hamouda *et al.*, 2010) [5]. Similarly, nanoemulsion formulations combined with recombinant anthrax protective antigen and HIV gp120 elicited strong systemic and mucosal immune responses following intranasal administration in experimental animals (Bielinska *et al.*, 2007; Bielinska *et al.*, 2008) [2, 1]. Furthermore, intranasal administration of W805EC formulated with hepatitis B surface antigen enhanced antigen-specific immune responses without inducing significant local or systemic adverse effects in mice, rats, guinea pigs, and dogs (Makidon *et al.*, 2008) [9]. Studies in ferrets also demonstrated enhanced humoral immunity and antigen-sparing effects when nanoemulsion-adjuvanted influenza vaccines were administered intranasally (Hamouda *et al.*, 2011) [6].

The increasing demand for safer and more effective vaccine adjuvants has intensified research into nanoemulsion-based delivery systems. In veterinary vaccinology, the development of stable, biocompatible, and immunologically effective adjuvants remains a critical requirement, particularly for recombinant and subunit vaccines that often exhibit limited intrinsic immunogenicity. Despite the promising potential of nanoemulsion adjuvants, their successful application depends on thorough physicochemical characterization and safety evaluation.

Therefore, the present study was undertaken to develop a novel mineral oil-based nanoemulsion using a sonication-assisted emulsification technique and to characterize its physicochemical properties, including particle size distribution, zeta potential, surface morphology, and thermodynamic stability. In addition, the safety of the formulated nanoemulsion was evaluated in guinea pigs to assess its suitability as a potential vaccine adjuvant for veterinary applications.

2. Material and methods

2.1 Preparation of Nanoemulsion

A mineral oil-based nanoemulsion (NE) was prepared using soyabean oil and span20 surfactants obtained from the Quality Control and Quality Assurance (QC&QA) Unit. The oil phase was prepared by mixing the required volume of soyabean oil with a lipophilic surfactant and heating the mixture at 70 °C for 5 min. Simultaneously, the aqueous phase was prepared by dissolving the Tween-80 surfactant in distilled water.

The aqueous phase was gradually added to the oil phase under continuous stirring to obtain a coarse emulsion. The

resulting emulsion was subjected to ultrasonic homogenization using a probe sonicator (Q500, Qsonica, USA; 20 kHz). Sonication was performed for 6 cycles at 20 sec with 10 sec break with progressive increases in amplitude. To minimize heat-induced instability, the emulsion was maintained in an ice bath throughout the sonication process. The resulting nanoemulsion was stored at 4 °C until further characterization.

2.2 Stability under Different Storage Conditions

To evaluate storage stability, nanoemulsion samples were maintained at 4 °C for 6 months, -20 °C for 3 months, and -80 °C for 1 month. Samples were periodically examined for changes in appearance, phase separation, flocculation, creaming, or demulsification.

2.3 Thermodynamic Stability Studies

The thermodynamic stability of the nanoemulsion was assessed by centrifugation, autoclaving, and dilution tests. For centrifugation studies, samples were centrifuged at 3000 rpm for 30 min at 4 °C and examined for phase separation, flocculation, creaming, or demulsification.

Autoclave stability was evaluated by exposing the nanoemulsion to 121 °C at 15 psi for 15 min. Following sterilization, samples were visually inspected for changes in physical appearance and phase stability.

Dilution stability was determined by diluting the nanoemulsion with phosphate-buffered saline (PBS) at dilution ratios of 1:25, 1:50, 1:100, 1:200, and 1:1000. The diluted samples were examined for dispersibility, homogeneity, and phase separation.

2.4 Surface Morphology

The morphology of the nanoemulsion droplets was examined using Field Emission Scanning Electron Microscopy (FESEM) at the Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bengaluru, India. A small volume of nanoemulsion was mounted on a specimen stub, air-dried, and examined under FESEM according to standard operating procedures.

2.5 Particle Size Analysis

The particle size distribution of both plain nanoemulsion and antigen-loaded nanoemulsion formulations was determined using a laser diffraction particle size analyzer (Mastersizer 2000, Malvern Instruments, UK). Samples were dispersed in Tris-NaCl buffer prior to analysis. Measurements were performed in triplicate, and the mean particle diameter was recorded.

2.6 Zeta Potential Analysis

The surface charge of the nanoemulsion droplets was determined using a Zetasizer Nano ZS instrument (Malvern Instruments, UK). Prior to measurement, samples were diluted in Tris-NaCl buffer. All measurements were performed in triplicate.

3. Results

3.1 Preparation of Nanoemulsion by Sonication method

The Nanoemulsion samples were prepared by sonication method as described in methods. The samples were prepared by using mineral oil & lipophilic, hydrophilic surfactant after standardizing the volume of surfactant, the stability of Nanoemulsion was tested in QC&QA lab.

3.2 Characterization of Nano-emulsion

Prepared samples were kept at various temperature and at various time interval like 4 °C for 6 months, -20 °C for 3 months, -80 °C for one month and that samples were observed frequently for phase separation, demulsification, and results of this study revealed no phase separation, (Fig.1A). The pH of that sample was checked at each time of shifting the sample from freezing to thawing. The result showed that pH (7.4 ± 0.2) of the sample was not changed by storing at various temperatures.

Therefore the prepared formulations were tested for thermodynamic stability study by centrifugation at 3000 rpm for 30 mins at 4 °C and autoclaved at 121 °C for 15 mins at 15 lbs pressure. The stability of that Nano-emulsion was good and there was no phase separation or flocculation

before and after centrifugation (Fig.1B) as well as in autoclaving (Fig.1B). The samples were diluted with PBS buffer at various dilutions like 1:25, 1:50, 1:100, 1:200 and 1:1000 were showing good dispersion (Fig.1C).

The emulsion were viewed under 40X were showing few micron size oil droplets, and its background were showing equal distribution of nano size oil droplets (Fig. 1D). The morphology of the nanoemulsion in Field Emission-Scanning Electron Microscope (FE-SEM) was found to be spherical and size ranging from 100-400nm (Fig.1E).

The mean droplet size of the Nano-emulsion preparation was found to be around 185.6 nm and the PDI was 0.245 in Zeta Sizer ® 3000-HS. The size distribution of the Nano-emulsion is shown in Table 1, Fig. 1F. The average zeta potential of the Nano-emulsion was 75.9 mV (Table.1).

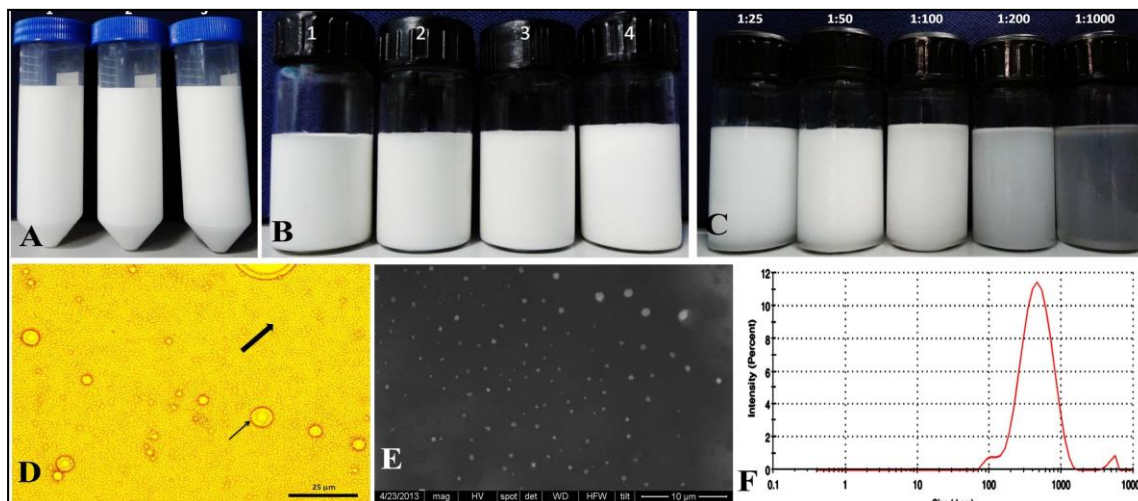


Fig. 1. Characterization of in-house prepared nanoemulsion for stability, dispersibility and morphology.

Representative figures are shown. A. Storage stability: Sample 1, 2 and 3 represent storage at 4 °C for 6 months, -20 °C for 3 months and -80 °C for one month, respectively. B. Suspension stability following centrifugation at 3000 rpm for 30 min at 4 °C [sample 1 (control) and 2 (spinning)] and autoclaving [sample 3 (control) and 4 (autoclaving)]. C. Dispersibility in PBS at different dilutions such as 1:25, 1:50, 1:100, 1:200 and 1:1000. D. Micrograph of NE at 40 X magnification showing a few micron size oil droplets (thin arrow). Background shows distribution of nano-size oil droplets (Bold arrow). E. Micrograph of NE droplets showing spherical morphology under Field Emission-Scanning Electron Microscope. F. Size distribution of NE showing maximum peak around 400 nm (Malvern Mastersizer 2000, Malvern Instruments, UK).

4. Discussion

Nanoemulsion-based adjuvants have emerged as promising vaccine delivery systems because of their ability to enhance antigen uptake, improve antigen presentation, and stimulate both humoral and cell-mediated immune responses. In the present study, a mineral oil-based nanoemulsion was successfully formulated using a combination of hydrophilic and lipophilic surfactants through ultrasonic emulsification. The formulated nanoemulsion exhibited desirable physicochemical characteristics, including long-term stability, nanoscale particle size distribution, and spherical morphology, supporting its suitability as a potential vaccine adjuvant.

The stability of nanoemulsions is a critical factor influencing their practical application in vaccine formulations. In the present study, the nanoemulsion remained stable under different storage conditions and exhibited no evidence of phase separation, flocculation, creaming, or demulsification following centrifugation and autoclaving. Similar findings have been reported that the ultrasonication-generated nanoemulsions had efficient surfactant coverage at the oil-water interface contributed to enhanced kinetic stability and prolonged shelf life (Ganesan *et al.*, 2023) [4]. The observed stability indicates that the surfactant system employed in the present formulation effectively prevented droplet aggregation during storage and processing.

Particle size and size distribution play crucial roles in determining the stability and biological performance of nanoemulsion-based vaccine adjuvants. The formulated nanoemulsion exhibited a mean droplet size of approximately 185.6 nm with a polydispersity index (PDI) of 0.245, indicating a relatively homogeneous distribution. Nanoemulsions with PDI values below 0.3 are generally considered to possess acceptable uniformity and physical stability. Similar observations were reported by Ganesan *et al.*, (2023) [4], who developed ultrasonication-based nanoemulsions with narrow particle size distributions and enhanced storage stability. The relatively low PDI obtained in the present study suggests efficient droplet formation during the sonication process and reduced susceptibility to coalescence.

Zeta potential is commonly used to evaluate colloidal stability. The zeta potential values of the present nanoemulsion ranged from -2.08 to $+3.28$ mV. Although these values were relatively low, the formulation remained physically stable throughout the study period. This finding suggests that steric stabilization rather than electrostatic repulsion was the primary mechanism responsible for maintaining stability. Similar observations have been reported for sterically stabilized nanoemulsions, where low absolute zeta potential values did not adversely affect long-term stability because of effective surfactant-mediated interfacial protection (Khan *et al.*, 2024; Herneisey *et al.*, 2023) [8, 7].

Morphological analysis by FESEM revealed predominantly spherical droplets ranging from 100 to 700 nm in diameter. Spherical morphology is a characteristic feature of stable nanoemulsion systems and is considered advantageous for enhanced dispersion and cellular interaction. Comparable morphological characteristics have been reported in

nanoemulsion-based delivery systems and vaccine nanoadjuvants, where spherical nanoparticles exhibited improved physicochemical stability and biological performance (Sun *et al.*, 2024; Filipić *et al.*, 2023) [12, 3]. The growing interest in nanoemulsion-based adjuvants is primarily attributed to their ability to improve antigen delivery and immune stimulation. Recent studies have demonstrated that nanoemulsion formulations facilitate efficient uptake by antigen-presenting cells, promote lymphatic transport, and enhance both humoral and cellular immune responses (Nooraei *et al.*, 2023; Zhao *et al.*, 2023) [10, 15]. These characteristics are particularly important for recombinant and subunit vaccines, which often require potent adjuvants to overcome their limited intrinsic immunogenicity. Therefore, the favorable physicochemical characteristics observed in the present study suggest that the formulated nanoemulsion could serve as an effective immunomodulatory platform for vaccine delivery.

Table 1: Size distribution and Zeta potential of the Nanoemulsion measured in Malvern Zeta sizer

Size distribution of Nanoemulsion was measured by using laser diffraction method using Malvern Mastersizer 2000		Zeta potential (mV) of the Nanoemulsion measured in Malvern Zeta sizer	
Intensity (%)	Size (nm)	Counts (number of droplets)	Zeta potential (mV)
2.6	103.3	34754	15.2
3.1	91.40	29552	58.6
3.7	95.46	26312	62.6
8.6	132.1	28655	88.1
9.7	134.0	36475	95.3
19.8	252.2	34892	97.5
26.33	491.35	49531	114.0

5. Conclusion

Overall, the present findings demonstrate that sonication-based emulsification successfully generated a stable mineral oil-based nanoemulsion with desirable physicochemical properties. The formulation maintained its stability under various stress conditions and exhibited particle size characteristics suitable for vaccine applications. These findings justify its further evaluation as a vaccine adjuvant, including studies on antigen-loading efficiency, cytokine responses, cellular immunity, and protective efficacy in target animal species.

6. Declaration Conflict of Interest

On behalf of all author declare that no conflict of interest with the publication

7. Ethics Statement

The animals used in this study were in accordance with the ethics of the IVRI, Izatnagar institutional animal ethics committee (IAEC).

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