The Development of Self-Emulsifying Oil-in-Water Emulsion Adjuvant and an Evaluation of the Impact of Droplet Size on Performance

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ABSTRACT: Microfluidization is an established technique for preparing emulsion adjuvant formulations for use in vaccines. Although this technique reproducibly yields high-quality stable emulsions, it is complex, expensive, and requires proprietary equipment. For this study, we developed a novel and simple low shear process to prepare stable reproducible emulsions without the use of any proprietary equipment. We found this process can produce a wide range of differently sized emulsions based on the modification of ratios of oil and surfactants. Using this process, we prepared a novel 20-nm-sized emulsion that was stable, reproducible, and showed adjuvant effects. During evaluation of this emulsion, we studied a range of emulsions with the same composition all sized below 200; 20, 90, and 160 nm *in vivo* and established a correlation between adjuvant size and immune responses. Our studies indicate that 160-nm-sized emulsions generate the strongest immune responses.
Keywords: vaccines; vaccine adjuvants; squalene; emulsion; particle size; formulation; self-emulsifying; physical characterization

INTRODUCTION

Vaccine adjuvants are components added to subunit or recombinant vaccines to raise the overall immunogenicity of poorly immunogenic antigens. In addition to enhancing and sustaining immune responses, adjuvants help in reducing the antigen dose per vaccine, reduce the frequency of vaccination, enhance the breadth of immune responses, and improve the immunological memory associated with the vaccine.[1] Adjuvants are composed of a large class of compounds ranging from inorganic aluminum salts to semisynthetic lipid-based systems such as emulsions, to naturally derived compounds like monophosphoryl lipid A. Despite the extensive discovery effort and inclusion of adjuvants in a number of marketed vaccines, the exact mechanism of action of many adjuvants is still under investigation. Among the adjuvants added in commercial vaccines, the oil-in-water (o/w) emulsion MF59 (Novartis Vaccines) was the first lipid particulate adjuvant to be successfully included in a commercial influenza vaccine. MF59 is produced by microfluidization, which is a well-established technique routinely used in pharmaceutical manufacturing.[2]

Although microfluidization has successfully produced o/w emulsion adjuvants such as MF59 and AS03 (GlaxoSmithKline), both at droplet size around 160 nm size, this technique has a limitation in droplet size reduction. Sizes less than 45 nm are difficult to produce by such conventional techniques, as the energy required to reduce the droplets that small is very high. Hence, using the established methods, the sizes that could be prepared are between 45 and 200 nm. Techniques such as microfluidization and homogenization are expensive, complex, and require proprietary equipment that is difficult to maintain. To generate stable small-sized emulsions less than 30 nm, we felt the need to develop a novel process that could address two issues: (1) can a single process resolve issues related to conventional techniques in terms of expense, high shear, complexity, and maintenance and (2) can this process generate emulsions less than 30 nm in size?

While exploring for low shear processes to produce stable o/w emulsions, we identified the approach of self-emulsifying drug delivery systems (SEDDs) routinely used in pharma for oral delivery of lipophilic drugs (e.g., Neoral® for cyclosporine delivery). SEDDs are isotropic mixtures of oil and surfactant that form fine o/w emulsions when diluted with an aqueous phase under conditions of gentle agitation.[3] They are specific to the nature of oil and surfactant, ratio of oil and surfactant, and the temperature needed for emulsification.[4] SEDDs are most commonly used for oral drug delivery and allow the emulsification to occur in the gut, minimizing the amount of shear needed to produce these fine oil droplets. Neoral is a microemulsion preconcentrate that emulsifies in the body to produce fine emulsion droplets below 100 nm in size.[5] The higher hydrophilie lipophile balance value of surfactants and the higher concentration of hydrophilic surfactants in the system make Neoral a type IIIb lipid formulation and help in generating self-emulsifying smaller-sized droplets.[6,7] Applying this concept, we designed a novel process for formulation of fine o/w emulsions without shear, where we mixed the oil and the surfactants and then introduced them in an aqueous phase with mild heating and stirring. This simple process utilizes minimal shear, is inexpensive, requires no proprietary equipment, and generated a

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range of emulsions that had different sizes based on the modification of ratios of oil and surfactants.

Although there is a lack of literature focusing on the effect of droplet size of emulsion adjuvants on overall vaccine responses, certain key features during the development of the o/w emulsion adjuvant MF59 (Novartis Vaccines) explains the rationale for the size of the final formulation. The size of MF59 (160 nm) was found to be important during its development where nanosized emulsions elicited better immune responses than micron-sized emulsions. Additionally, the nanosize enabled sterile filtration of the emulsion with a 0.22-μm filter, making terminal sterilization a key attribute of parenteral formulations feasible.[8] Because of the limitations of particle size reduction by microfluidization, no thorough investigation to find the optimal size to generate the most potent response exists. As adjuvant development comprises a number of delivery systems similar to pharma (emulsion, polymeric particles, and liposomes), there is a tendency to extrapolate the results of drug delivery to vaccines. Although majority of size-related studies for vaccine adjuvants have been performed for polymeric particles, there are few studies that have been performed for other classes of adjuvants as well. A study by Li et al.[9] showed that smaller-size aluminum nanoparticles exhibited improved immune responses and tolerance in comparison to larger micron-sized particles. In case of liposomes, the particle size controls quality and type of response rather than controlling the magnitude of responses, for example, Th1 versus Th2 differentiation.[10,11] For polymeric particles, it is difficult to make a correlation between size and responses; depending on desired outcome, type of polymer, and antigen used, the results can be different.[12] Thus, based on the published reports, it is clear that each class adjuvant may have an ideal size that is different than another.

In this study, we describe a novel process to develop 20-nm-sized emulsions. Using the composition of the 20-nm-sized emulsion, emulsions with 90 and 160 nm size were prepared by conventional techniques (homogenization and microfluidization). These emulsions were subsequently evaluated *in vivo* to establish their potency and to compare the effect of droplet size on immune responses with a model antigen ovalbumin and with flu vaccine.

MATERIALS AND METHODS

Materials

Squalene oil, sorbitan trioleate (Span 85), and phosphate-buffered saline (PBS) were obtained from Sigma–Aldrich (St. Louis, MO), and polysorbate 80 (Tween 80) was obtained from Acros Organic (Geel, Belgium). Millipore MilliQ deionized water was used for formulation and assay procedures and 100 mM citrate buffer was acquired from Teknova (Hollister, CA). Reagents for the gel clot assay to test the endotoxin level of the formulations were procured from Associates of Cape Cod (East Falmouth, MA). Goat antimouse OVA HRP (horseradish peroxidase) conjugate secondary antibody for ELISA (enzyme-linked immunosorbent assay) was obtained from Sigma–Aldrich and TMB substrate was procured from KPL (Gaithersburg, MD). Antibodies, dyes, and compensation beads for T-cell analysis were obtained from BD Biosciences (Franklyn Lakes, NJ). Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Invitrogen (Carlsbad, CA).

Antigens for Immunizations

To establish proof of concept, ovalbumin (OVA) procured from Worthington (Lakewood, NJ) was used as a model antigen in the initial couple of studies. For the next study, equal amounts of trivalent-inactivated influenza vaccine (TIV) antigens were used: H1N1 A/California/7/09, H3N2 A/Texas/50/2012, and B/Massachusetts/2/2012 at 0.1 μg dose each. The trivalent vaccine contains purified subunit antigens and is standardized for hemagglutinin content by single radial immunodiffusion as recommended by regulatory authorities.

Screening of Squalene, Span 85, and Tween 80 Ratios for Self-Emulsification

Self-emulsification is sensitive to the ratio of oil and surfactants added to the aqueous phase. Hence, to formulate small emulsions of less than 30 nm, identification of the correct ratio of oil and surfactant was essential. Using the components of MF59, we studied different concentrations of squalene, Span 85, and Tween 80 to obtain a self-emulsifying ratio that can produce emulsion droplets in the size range of 20–30 nm (Fig. 1). Thirty-six different ratios of oil and surfactants were mixed overnight at room temperature (RT), and emulsified the next day with deionized water at a ratio of 1:20. These mixtures were heated at 50°C for half an hour and sized on Malver Zetasizer (Malvern Instruments, Malvern, UK) by dynamic light scattering (DLS).

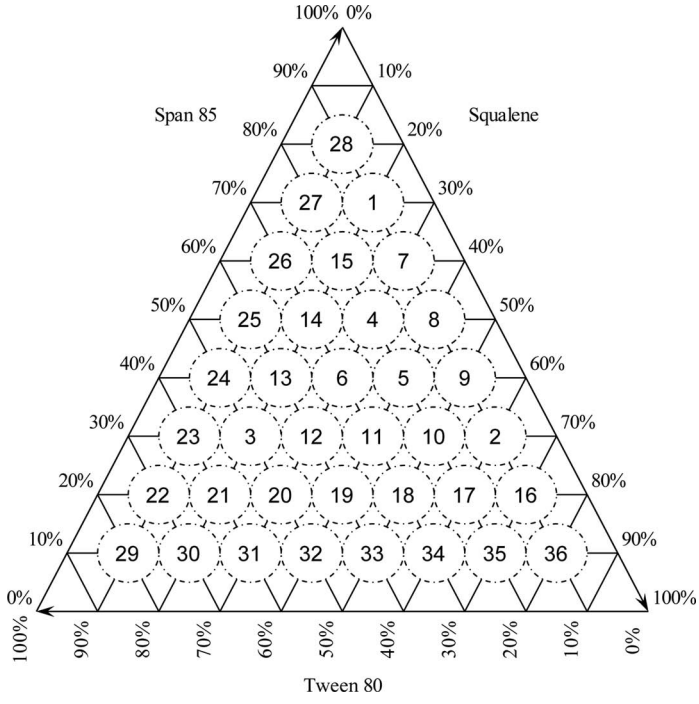

Figure 1. Ternary phase diagram of ratios of squalene, Span 85, and Tween 80. Components of MF59 were analyzed for their ability to self-emulsify at different concentrations. A ternary phase diagram was generated to analyze trends related with ratios of components and droplet sizes.

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