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Monitoring the effects of component structure and source on formulation stability and adjuvant activity of oil-in-water emulsions

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ABSTRACT

Oil-in-water emulsions have shown promise as safe and effective adjuvant formulations for vaccines. In particular, formulations consisting of metabolizable oils such as shark-derived squalene and detergents such as egg phosphatidylcholine have been used to produce stable vaccine emulsion formulations. However, there is an emphasis in pharmaceutical regulatory bodies on using synthetic or plant-derived components from sustainable sources instead of animal-derived components. This study compares the physicochemical properties and biological efficacy of emulsions consisting of oil and detergent components from animal, plant, and synthetic sources. In particular, effects of component structure and source on emulsion stability and biological activity are examined. It is shown that oil-in-water emulsions using animal-derived components can be substituted with synthetic or plant-derived materials while still exhibiting satisfactory physicochemical and biological properties.

1. Introduction

A great deal of attention has been recently directed towards the development of new adjuvant formulations. These adjuvant systems are typically a combination of an immunostimulatory molecule and a delivery system such as alum salt particulates, emulsions, or liposomes. They are used in conjunction with vaccine antigens to potentiate immune responses (humoral and/or cell mediated) or to reduce the amount of antigen needed to elicit a given response [1–3]. Specific vaccine antigens may be more effective with one adjuvant system as compared to another [2]. Currently, alum is the sole FDA-approved adjuvant delivery system [4,5]. Although alum-based adjuvants have exhibited an acceptable safety profile for many years, they have been known to induce local reactogenicity [6], and aluminum is a known neural toxin [7]. Therefore, a need exists to develop additional adjuvant systems that are both safe and effective.

Various adjuvant formulations other than alum are being researched, including aqueous formulations [4,8], emulsions [9,10], liposomes [11,12] and other small particles [9,13]. Stable emulsions (SE) are adjuvant delivery systems likely to garner FDA approval (approval in Europe has already been achieved for the emulsion formulation MF59) [9] and there are multiple ongoing clinical trials utilizing emulsion delivery systems [1,9]. Typically, SE formulations are composed of metabolizable oil droplets, such as squalene, stabilized by one or a combination of natural and synthetic detergents in a buffered aqueous phase (oil-in-water or o/w) [14]. The converse is also an option, where water droplets are stabilized in an oil phase (water-in-oil or w/o). Squalene and phosphatidylcholine (PC), a natural detergent commonly used in SE formulations, are often from animal sources such as shark liver and chicken egg yolk, respectively. For regulatory and sustainability purposes it would be more desirable to utilize plant-derived or synthetic sources if SE is to become a widely used adjuvant delivery system. These plant- or synthetic-based systems must have stability and immunopotentiating profiles similar to traditional animal-derived emulsions while remaining cost effective.

This study aims to examine the stability of SE formulations consisting of oil and detergent components from animal, plant, and synthetic sources. Specifically, olive-derived squalene and the plant-derived Miglyol 810 oil were compared to shark-derived squalene. The contribution of egg yolk PC to emulsion stability as compared to soy-derived PC or synthetic PC was also investigated. Furthermore, the effects of the detergents polysorbate 80 (Tween 80®) vs. Poloxamer 188 (Pluronic F68®) on emulsion stability were evaluated. Finally, in vivo antibody responses were compared using the different emulsion components. Together, physicochemical and

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biological data from these emulsions indicate that plant-based formulations can be made that exhibit no significant reduction in adjuvanticity or stability compared to the animal-based formulations.

2. Materials and methods

2.1. Materials

Shark liver squalene was purchased from Sigma–Aldrich (St. Louis, MO). Olive-derived squalene was purchased from Wilshire Technologies, Inc. (Carlsbad, CA) and NutriScience Innovations (Trumbull, CT). Miglyol 810, a capric/caprylic acid-based metabolizable oil, was obtained courtesy of Sasol Germany GmbH (Witten, Germany). Egg yolk PC, soy PC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine, 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine, and 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Soy PC was also obtained from Larodan (Malmo, Sweden), ChemImpex (Wood Dale, IL), Lipoid (Ludwigshafen, Germany), and the American Lecithin Company (Phospholipon 90G; Oxford, CT). Polysorbate 80 (Tween 80), made from a non-animal source, was purchased from J.T. Baker (Phillipsburg, NJ) and Pluronic F68 NF prill surfactant was obtained courtesy of BASF (Florham Park, NJ). The vaccine antigen Leish-111f was prepared as described previously [15]. The buffer components monobasic and dibasic ammonium phosphate were obtained from Riedel-de Haën (Seelze, Germany) and Spectrum Chemical (Gardena, CA). Glycerol and α-tocopherol were purchased from Sigma–Aldrich and Spectrum Chemical (Gardena, CA), respectively. Distilled water was deionized with a Barnstead E-pure for use in all formulations. Water for injection (WFI) was obtained from Abbot Laboratories (North Chicago, IL).

2.2. Emulsion formulation

All emulsion formulations were made by mixing a buffered aqueous phase and an oil phase with a Silverson Heavy Duty Laboratory Mixer Emulsifier (3/4 in. tubular square hole high shear screen attachment; East Longmeadow, MA) at ~5000–10,000 rpm for several minutes, and submitting the mixture to high-pressure homogenization using the Microfluidics M110-EH (Newton, MA) for 12 passes at ~30,000 psi. The buffered aqueous phase was prepared by combining 20 ml of 250 mM ammonium phosphate (pH 5.1 ± 0.05), detergent (either 0.18 g Pluronic F68 or 1.06 g Tween 80), and 4.54 g glycerol in 156.4 ml of ultrapure water. The oil phase was prepared by dissolving 3.8 g of PC into 20.2 ml of squalene or Miglyol and 0.12 g of α-tocopherol. α-Tocopherol was omitted from some formulations as indicated. The oil phase was then sonicated in a VWR 75D or 50HT (West Chester, PA) sonicating water bath at ~50 °C until the PC was fully dissolved with the exception of 1 or 2 formulations where some PC remained undissolved even after several hours of sonication. The buffered aqueous phase was then combined at 90% (v/v) with the oil phase. Final component concentrations are listed in Table 1. All formulations were stored at 2–8 °C.

2.3. Stability analysis

Emulsion particle size and stability were determined via dynamic light scattering (DLS) with the Malvern Instruments (Worcestershire, UK) Zetasizer Nano-S. The hydrodynamic diameter, *d*, was calculated using the measured translational diffusion, *D*, according to the equation $d = k_B T/(3\pi\mu D)$, where T is the temperature, μ is the fluid viscosity, and k_B is Boltzmann's constant. The diameter obtained with this technique is that of a sphere with the same translational diffusion coefficient as the particle being measured. Formulations were analyzed on the day of manufacture and at periodic intervals thereafter; in general, analysis was carried out 1 week, 2 weeks, 1 month, and 3 months after manufacture by combining 5 μl emulsion with 0.5 ml WFI in 1.5 ml polystyrene disposable cuvettes. All DLS measurements were made three times on each of three separate aliquots. In addition, formulations were visually inspected for phase separation and agglomeration prior to light scattering analysis.

Table 1
Emulsion component concentrations

Component	Weight or volume %	Concentration (mg/ml)
Oil	10.00% (v/v)	85.84
Glycerol	1.80% (v/v)	22.72
α-Tocopherol	0.05% (w/v)	0.5
PC	1.92% (w/v)	19.16
Pluronic F68 or Tween 80	0.09% or 0.53% (w/v)	0.91 or 5.32
25 mM ammonium phosphate buffer	88.20% (v/v)	

2.4. High-performance liquid chromatography (HPLC) of squalene

Squalene purity was analyzed by HPLC. The HPLC system consisted of an Agilent 1100 (Santa Clara, CA) equipped with a Waters Atlantis C18 5 μm column (4.6 mm × 250 mm; Milford, MA) and an ESA Biosciences Corona charged aerosol detector (Chelmsford, MA). The method consisted of a 50 μl injection with a linear gradient from 100% to 10% mobile phase A over 45 min and a column temperature of 30 °C. Mobile phase A consisted of 75:15:10 (v/v/v) methanol:chloroform:water with 20 mM ammonium acetate and 1% acetic acid. Mobile phase B consisted of 50:50 (v/v) methanol:chloroform with 20 mM ammonium acetate and 1% acetic acid. Samples were prepared in mobile phase B at 4 mg/ml.

2.5. In vivo antibody assay

In vivo experiments were carried out using BALB/c mice. The recombinant polyprotein vaccine Leish-111f [16,17] was used as antigen in emulsion formulations consisting of squalene and PC components from both animal and plant sources. Mice were injected subcutaneously at the base of the tail with 100 μl of antigen solution, which contained 10 μg of antigen and 20 μl of emulsion formulation. Mice were bled 7 days after injection and Ab response to Leish-111f was evaluated using enzyme-linked immunosorbent assays (ELISAs) for IgG1 and IgG2a with five-fold serial dilutions of serum samples starting at 1:100. Mice were boosted at day 21 and bled again at day 28 to determine Ab response. Each test group consisted of 4 or 5 mice.

3. Results

Emulsion droplet size is an indicator of stability since it is thermodynamically more favorable for emulsions to minimize interfacial contact by decreasing droplet surface area/volume ratio until phase separation. After examination of visual appearance, emulsion particle size from each sample was analyzed periodically over at least 3 months using DLS. Within the scope of this study, an *unstable* emulsion was defined as visible evidence of phase separation of the oil and aqueous phases and/or oil droplet diameter (particle size) change of more than 50% of the original particle size

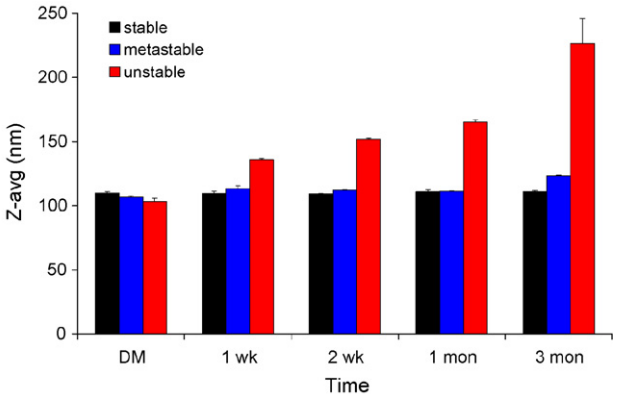

Fig. 1. The average particle size of emulsions determined by DLS is an indicator of stability. Particle size is shown as Z-avg, which is the mean scattering intensity particle size measurement obtained from DLS. In general, emulsions were tested on the day of manufacture (DM) and at periodic timepoints thereafter as indicated over 3 months. Emulsions are classified as unstable if they exhibit more than 50% particle size growth over 3 months or more than 20% growth over the first week, metastable if they exhibit 11–50% particle size growth over 3 months, and stable if particle size grows by 10% or less over at least 3 months.

as measured on the day of manufacture and over the next 3 months. In addition, emulsions were considered unstable if particle size grew more than 20% over the first week. Some emulsions show a consistent trend of mild particle size growth over the 3-month period (between 11% and 50%) and these are herein characterized as *metastable*. Emulsions showing 10% change or less in particle size over 3 months or more are classified as *stable* in this study. A few of these stable emulsions exhibited one particle size measurement above the 10% range but measurements in subsequent weeks were all found to be within the 10% range. Examples of particle size results of stable, metastable, and unstable emulsions are shown in Fig. 1.

The structures of the various oil, PC, and surfactant components used in this study are shown in Fig. 2. The structure, source, and purity of the oil component in oil-in-water emulsions are important factors in determining emulsion stability (Fig. 3). The stability of emulsions prepared with oils from different sources with egg

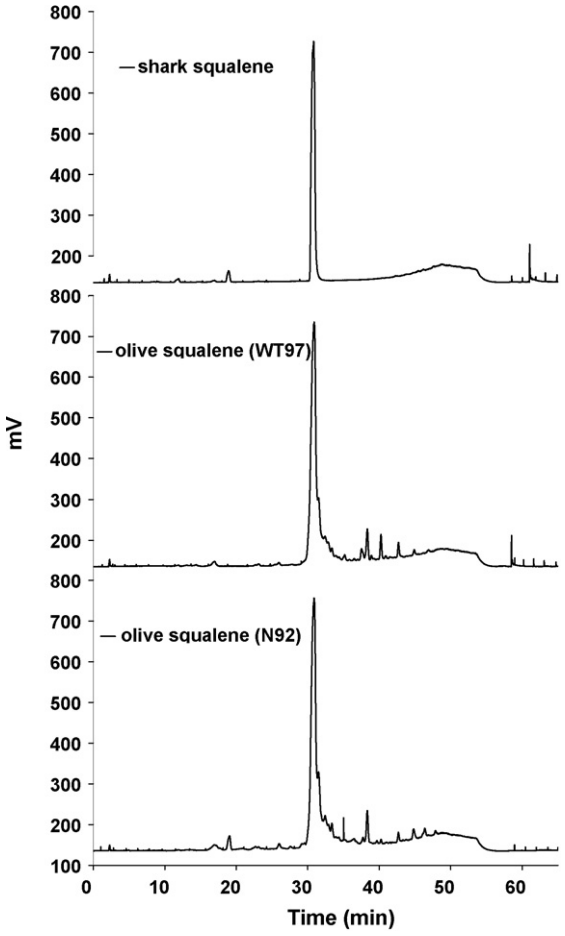

Fig. 3. HPLC detection of squalene impurities in shark and olive squalene. Olive squalene has several unique peaks indicating various impurities. WT97: obtained from Wilshire Tech. (97% purity); N92: obtained from Nutriscience (92% purity).

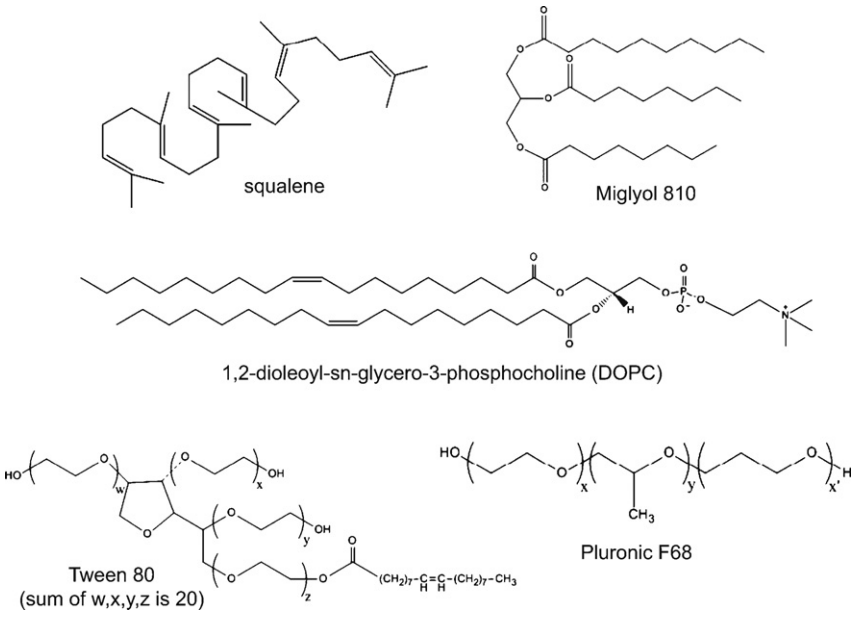

Fig. 2. Chemical structures of squalene, Miglyol 810 (representative triglyceride), DOPC (a common phospholipid found in both egg and soy PC), Tween 80, and Pluronic F68.

Table 2
Effect of oil content on emulsion stability with egg PC and Pluronic F68 as emulsifiers

Oil	PC	Cosurfactant	Vit E	Stability at 3 months	Particle size[a] (nm)
Shark squalene	Egg	Pluronic F68	Y	Stable	109
Shark squalene	Egg	Pluronic F68	Y	Stable	104
Shark squalene	Egg	Pluronic F68	Y	Stable	118
Shark squalene	Egg	Pluronic F68	N	Stable	101
Shark squalene	Egg	Pluronic F68	N	Stable	107
Olive squalene (N85)	Egg	Pluronic F68	Y	Stable	124
Olive squalene (WT97)	Egg	Pluronic F68	Y	Stable	117
Olive squalene (WT97)	Egg	Pluronic F68	N	Metastable[b]	107
Olive squalene (WT97)	Egg	Pluronic F68	N	Stable	108
Olive squalene (N92)	Egg	Pluronic F68	N	Stable	106
Miglyol 810	Egg	Pluronic F68	Y	Stable	115
Miglyol 810	Egg	Pluronic F68	N	Stable	110
Miglyol 810	Egg	Pluronic F68	N	Stable	113

All shark squalene, Miglyol 810, egg PC, and Pluronic F68 materials are from single sources as described in Section 2. Olive squalene from various sources was used as follows: N85: obtained from Nutriscience (85% purity); N92: obtained from Nutriscience (92% purity); WT97: obtained from Wilshire Tech. (97% purity).
[a] Measured on the date of manufacture.
[b] Metastable at 3-month measurement; however, at 6 months the particle size had dropped back within the stable range.

Table 3
Effect of oil content on emulsion stability with soy PC and Pluronic F68 as emulsifiers

Oil	PC	Cosurfactant	Vit E	Stability at 3 months	Particle size[a] (nm)
Shark squalene	Soy (A95)	Pluronic F68	Y	Stable	117
Shark squalene	Soy (A95)	Pluronic F68	Y	Stable	109
Shark squalene	Soy (Li98)	Pluronic F68	Y	Stable	97
Shark squalene	Soy (La98)	Pluronic F68	Y	Unstable	96
Shark squalene	Soy (C99)	Pluronic F68	Y	Unstable	95
Shark squalene	Soy (A95)	Pluronic F68	N	Stable	97
Shark squalene	Soy (A95)	Pluronic F68	N	Stable	99
Olive squalene (N85)	Soy (A95)	Pluronic F68	Y	Unstable	103
Olive squalene (WT97)	Soy (A95)	Pluronic F68	Y	Unstable	107
Olive squalene (WT97)	Soy (A95)	Pluronic F68	Y	Metastable	124
Olive squalene (WT97)	Soy (A95)	Pluronic F68	N	Stable	104
Olive squalene (WT97)	Soy (A95)	Pluronic F68	N	Stable	106
Olive squalene (WT97)	Soy (ALC94)	Pluronic F68	N	Stable	103
Olive squalene (N92)	Soy (ALC94)	Pluronic F68	N	Metastable	101
Olive squalene (N92)	Soy (A95)	Pluronic F68	N	Stable	99
Miglyol 810	Soy (A95)	Pluronic F68	Y	Metastable	167
Miglyol 810	Soy (A95)	Pluronic F68	N	Unstable	112
Miglyol 810	Soy (A95)	Pluronic F68	N	Metastable	105
Miglyol 810	Soy (A99)	Pluronic F68	N	Unstable	108

All shark squalene, Miglyol 810, egg PC, and Pluronic F68 materials are from single sources as described in Section 2. Olive squalene and soy PC from various sources were used as follows: A95: obtained from Avanti Polar Lipids (95% purity); A99: obtained from Avanti Polar Lipids (99% purity); ALC94: obtained from American Lecithin Co. (94% purity); C99: obtained from ChemImpex (99% purity); La98: obtained from Larodan (98% purity); Li98: obtained from Lipoid (98% purity); N85: obtained from Nutriscience (85% purity); N92: obtained from Nutriscience (92% purity); WT97: obtained from Wilshire Tech. (97% purity).
[a] Measured on the date of manufacture.

PC and Pluronic F68 as emulsifiers is shown in Table 2. Overall, the olive-derived squalene and the capric/caprylic acid-based Miglyol 810 demonstrated comparable stability to the shark-derived squalene samples when egg PC and Pluronic F68 were used as detergents. However, Tables 2–4 indicate several plant-based samples that show less stability than the animal-based emulsions. For instance, there appears to be a higher incidence of less stable emulsions when shark squalene is replaced by olive squalene.

The structure and source of the phospholipid detergent component in oil-in-water emulsions is also an important contributor to emulsion stability. This is apparent by comparing the stability of emulsions prepared with egg-derived PC with those employing soy PC (Tables 2 and 3). In general, soy PC emulsions demonstrated decreased stability among all oils studied; the Miglyol-based emulsions were especially unstable (Table 3). Substituting the emulsion PC component with the single synthetic DOPC or a synthetic PC

Table 4
Effect of oil content on emulsion stability with synthetic PC and Pluronic F68 as emulsifiers

Oil	PC	Cosurfactant	Vit E	Stability at 3 months	Particle size[a] (nm)
Shark squalene	DOPC	Pluronic F68	N	Stable	107
Olive squalene (WT97)	DOPC	Pluronic F68	N	Metastable	106
Shark squalene	Synthetic mix[b]	Pluronic F68	N	Stable	98

All shark squalene, DOPC, and Pluronic F68 materials are from single sources as described in Section 2. Olive squalene was obtained from Wilshire Tech. (97% purity) (WT97).
[a] Measured on the date of manufacture.
[b] Synthetic PC mix contained various synthetic phosphatidylcholines at weight ratios designed to mimic natural egg PC content.

Table 5
Effect of oil content on emulsion stability with PC and Tween 80 as emulsifiers

Oil	PC	Cosurfactant	Vit E	Stability at 3 months	Particle size[a] (nm)
Shark squalene	Soy (A95)	Tween 80	Y	Stable	88
Shark squalene	Soy (A95)	Tween 80	N	Stable	92
Shark squalene	DOPC	Tween 80	N	Stable	99
Olive squalene (N92)	Egg	Tween 80	N	Stable	118
Olive squalene (N92)	Soy (A95)	Tween 80	Y	Stable	90
Olive squalene (WT97)	Soy (A95)	Tween 80	Y	Stable	92
Olive squalene (WT97)	Soy (A95)	Tween 80	Y	Stable	90
Olive squalene (WT97)	Soy (A95)	Tween 80	N	Stable	98
Miglyol 810	Egg	Tween 80	N	Stable	87
Miglyol 810	Soy (A95)	Tween 80	Y	Stable	113
Miglyol 810	Soy (A95)	Tween 80	N	Stable	92
Miglyol 810	Soy (A95)	Tween 80	N	Stable	102
Miglyol 810	Soy (A99)	Tween 80	N	Stable	92
Miglyol 810	Soy (ALC94)	Tween 80	N	Stable	89

All shark squalene, Miglyol 810, egg PC, DOPC, and Tween 80 materials are from single sources as described in Section 2. Olive squalene and soy PC from various sources were used as follows: A95: obtained from Avanti Polar Lipids (95% purity); A99: obtained from Avanti Polar Lipids (99% purity); ALC94: obtained from American Lecithin Co. (94% purity); N92: obtained from Nutriscience (92% purity); WT97: obtained from Wilshire Tech. (97% purity).

[a] Measured on the date of manufacture.

blend mimicking the content of egg PC demonstrated good stability in shark squalene emulsions (Table 4).

The stability of emulsions containing soy PC was greatly improved by using Tween 80 as coemulsifier instead of Pluronic F68 (Table 5). Indeed, all emulsions employing Tween 80 as emulsifier showed excellent stability profiles among all oil and PC components employed. Causes of emulsion stability are discussed in terms of the multiple interactions occurring at the oil/water interface (Fig. 4). It is determined that stable emulsions from non-animal source components may be produced using olive squalene or Miglyol 810 combined with soy or synthetic PC and Tween 80 as emulsifiers. Moreover, in vivo results indicate that substitution of animal-source oil and PC components with plant-derived material does not affect in vivo adjuvanticity as measured by antibody response in a mouse model (Fig. 5).

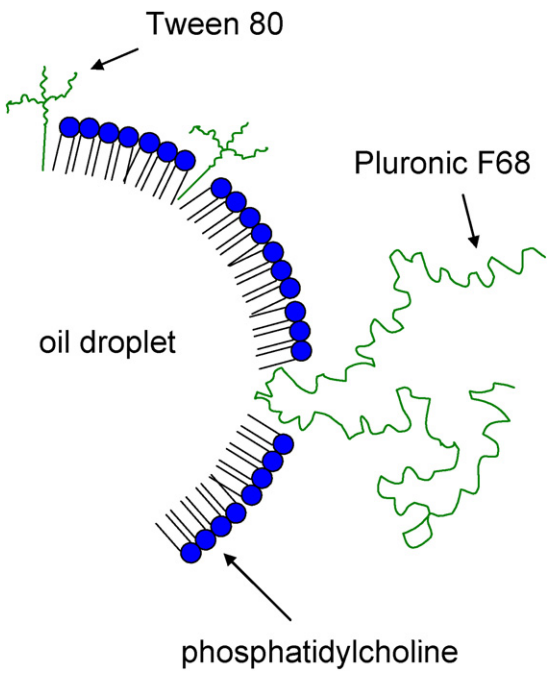

Fig. 4. Schematic representation of oil droplet interface. Pluronic F68 and Tween 80 are shown together for comparison; however, they were separately employed in the emulsion formulations of this study.

4. Discussion

4.1. Oil source

There appears to be a slight improvement in the stability of shark-derived squalene emulsions compared to olive-derived squalene emulsions (Tables 2–4). This discrepancy in stability may be due to differences in compound purity. Shark-derived squalene used in this study was at least 99% pure as stated by the manufacturer, whereas olive-derived squalene purity ranged from 85% to 97% depending on the manufacturer. Indeed, HPLC analysis revealed various unique peaks in the olive-derived squalene (Fig. 3). Impurities that are closely related to squalene such as cyclosqualene or hydrocarbon chains of different lengths vary significantly according to source [18]. Therefore, changes in squalene emulsion properties may be due to the amount and type of impurities. Obtaining olive squalene of higher purity would allow a more direct comparison between the shark and olive sources. In addition, more emulsions would need to be analyzed to establish a clear difference in emulsion stability between the two sources of squalene. Of course, oils other than squalene could also be suitable as the emulsion dispersed phase. Miglyol 810, comprised mostly of triglycerides of capric or caprylic acid and obtained from coconut and palmkernel oils, have exhibited desirable emulsion stability characteristics [19] and are of high purity [20]. In this study, olive squalene or Miglyol 810 were found to produce stable emulsions when certain combinations of emulsifiers were used. For regulatory and sustainability reasons, plant-derived oils are more desirable than animal-derived oils. Thus, a stable emulsion employing olive squalene or Miglyol 810 would be preferable to a shark squalene emulsion.

4.2. Phosphatidylcholine source

The phosphatidylcholine source appears to have a much more dramatic effect on emulsion stability than the oil source (Tables 2–4). Emulsions made with egg-derived PC are much more stable than those made with soy-derived PC. This discrepancy in stability may be attributable to several causes. Similar to the above discussion on oil purity, it appears that PC purity may be a factor in the emulsifying efficacy of egg PC vs. soy PC. PC purity is especially important in pharmaceutical formulations, considering that soy PC protein impurities were considered the cause of hypersensitivity reactions in some patients [21]. In general,

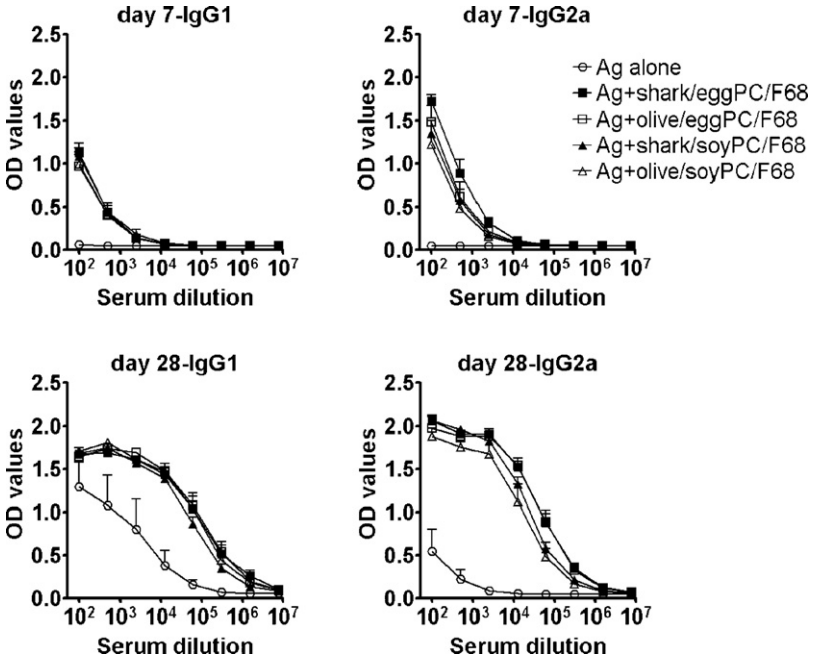

Fig. 5. Antibody response of mice injected with antigen (Ag) formulated in emulsions composed of squalene and PC from different sources. Significantly increased antibody titers were detected by ELISA in mice injected with Ag formulated in emulsion than those injected with Ag alone. In general, no significant difference in antibody titers is apparent among the different emulsions. Olive squalene and soy PC used in this experiment were obtained from Nutriscience (85% purity) and Avanti (95% purity), respectively, and all emulsions contained α-tocopherol The points shown are the mean OD values of 4 or 5 mice and the error bar is the standard error of the measurement.

the egg PC products used in this study were of a higher purity (99%) than the soy PC (95%). Thus, as might be expected, egg PC demonstrated significantly increased emulsion stability compared to soy PC. However, when higher purity soy PC (99%) was used (Table 3), an improvement in stability was not demonstrated, implying that other factors may be relevant. According to the manufacturers [22–24], soy and egg PC differ significantly in phospholipid chain length and saturation. While both PC mixtures primarily consist of 16- and 18-C phosphatidylcholine molecules, egg PC consists of twice as many of the shorter 16-C chains than soy PC, which in turn has a significantly higher percentage of 18-C chains. Moreover, the ratio of saturated/unsaturated PCs in the egg source is ∼0.8, whereas for soy it is ∼0.3. This increase in unsaturated lipids may cause soy PC molecules to be more fluid and disordered compared to egg PC. A more disordered lipid structure may indicate less efficient packing at the particle surface, decreasing the rigidity and stability of the interfacial film.

Another reason that lipid saturation is of importance is that emulsifiers, in general, have been reported to be more effective at emulsifying oils of the same type of structure (i.e. unsaturated vs. saturated) [14]. For example, when emulsifying the saturated, short-chain oil glyceryl trioctanoate, it was found that short-chain, saturated phospholipids were more effective than long-chain, unsaturated phospholipids [25]. Based on this reasoning, it may be somewhat surprising that soy-derived phosphatidylcholine is less effective as an emulsifier of squalene than egg-derived phosphatidylcholine, since soy PC has a higher content of unsaturated phospholipid and long acyl chain content and squalene is likewise a long-chain unsaturated hydrocarbon (Fig. 2). Alternatively, when employing Miglyol 810 as the dispersed phase, which consists mostly of saturated short-chain triglycerides, the situation is reversed and egg PC (with a higher saturated content than soy PC) would be expected to be the more effective emulsifier, which was indeed the case when Pluronic F68 was employed as cosurfactant (Tables 2 and 3).

To determine the importance of PC lipid composition in stabilizing emulsions, synthetic PCs were substituted as the PC source. Employing a synthetic 18:1 PC (DOPC) or a synthetic PC mixture mimicking egg PC content resulted in stable emulsions when shark squalene was used as the oil (Tables 4 and 5). It should be remembered that since egg PC is derived from animals, it would be preferable to use plant-derived soy PC or synthetic PC in pharmaceutical formulations. Finally, lipid oxidation does not appear to play a significant factor in emulsion stability on the timescale of this study since the presence of the antioxidant α-tocopherol did not exhibit a noticeable effect on stability (Tables 2 and 3).

4.3. Cosurfactant

It has been shown that using a mixture of emulsifiers (combining a hydrophobic surfactant with a hydrophilic surfactant) makes more stable emulsions compared to using a single emulsifier [14,21,26,27]. Presumably, this is because the presence of cosurfactant allows tighter packing at the interface, which gives a more stable detergent film [9,14,21,26]. An additional advantage conferred by cosurfactants is that they tend to decrease phospholipid hydrolysis [21]. The importance of interfacial packing is demonstrated by the fact that cosurfactants with similar hydrophobic chain lengths and molar concentrations have been shown to produce stable emulsions [14]. For example, the vaccine formulation MF59 employs a 1:1 molar ratio of Tween 80 and Span 85. Tween 80 and Span 85 are detergents consisting of the same hydrocarbon chain length and level of saturation, and complementary structures that optimize packing interactions [9,28,29]. However, the cosurfactant molecules used in the present study have a significantly larger area per molecule than the phospholipid molecules. Thus, it is possible that multiple phospholipid molecules are associated with each cosurfactant molecule. Presumably, the hydrophobic phospholipids partition largely into the oil phase while the cosurfactants partition largely into the aqueous phase with hydrophilic chains of extensive

length and maintain a hydrophobic anchor in the oil phase (Fig. 4).

In general, the ability of Pluronic F68 to induce emulsion stability was dependent on the sources of phospholipid and oil, while Tween 80 apparently stabilized all emulsions in which it was employed (Tables 2–5). While both Pluronic F68 and Tween 80 are non-ionic surfactants commonly used in pharmaceutical formulations, it is difficult to make a direct comparison between the two emulsifiers in this study since they were used at significantly different concentrations. This is due to the fact that the surfactant concentrations used here were based on those of previous adjuvant formulations known as MPL-SE (Pluronic F68) and MF59 (Tween 80) [9]. The higher surfactant concentration in emulsions containing Tween 80 may have been responsible for the increased stability. In any case, ongoing studies in our lab are seeking to correlate the significant differences in structure between the two surfactants to effects on emulsion stability.

4.4. Emulsion stability and heterogeneity

The manufacture and stabilization of emulsions of nanometer dimensions is important for several reasons. It has been demonstrated that, in general, smaller droplet emulsions are more stable over time than large droplet emulsions [9,21,25,30]. However, as emulsion stability decreases, larger droplets will be increasingly formed since a decrease in the surface area to volume ratio is energetically more favorable [14]. Furthermore, larger particles show increased rates of clearance from the body [31] and above 4 μm can even be biologically toxic [21]. In drug delivery applications, smaller droplets may also induce higher drug release rates from emulsion droplets due to an increase in droplet curvature and surfactant layer flexibility [32,33]. Besides droplet size, other formulation considerations (including stability effects) include oil and detergent composition and concentration [30,34], detergent HLB values [14], viscosity [9,14,27,30,32], particle surface charge [21,27,32], the interfacial tension between oil and water [30], temperature [25], buffer composition [35], and packaging [21].

The two basic mechanisms of emulsion instability include Ostwald ripening and coalescence, though coalescence may be preceded by flocculation, creaming, or sedimentation [14,27,36,37]. Ostwald ripening involves the diffusion of oil molecules from smaller droplets to larger droplets due to Laplace pressure differences [27,36]. However, this mechanism is unlikely when dealing with oils that are essentially insoluble in water, such as triacylglycerols or squalene [27,35–37]. Coalescence involves the disruption of the thin water layer between oil droplets as they merge into one droplet [14,36]. However, due to the complex interfacial phenomena involving multiple components, the complete mechanisms of emulsion stability or instability, interfacial packing of emulsifiers, and biological effects are often little understood [35,36]. This is especially true when dealing with pharmaceutical or vaccine formulations, which often include proteins, adjuvant molecules, and other excipients that further complicate emulsion structure and interactions. For example, the vaccine emulsion formulation MF59 has been shown to be effective and safe (despite local reactogenicity) [9,38–40], but the mechanism of action of MF59, including the amount of direct association existing between the antigen and the emulsion formulation, is unclear [9].

It is important to note that emulsion formulations are most likely not homogeneous solutions of uniform droplet size [21]. Instead, there are often phospholipid liposomes, detergent micelles, monomers, and varying droplet diameters present [21,37,41–43]. For example, it has been shown that a significant proportion (up to 20% or more) of particles in many phospholipid-stabilized emulsions are in fact liposomes [41,42,44]. Furthermore, contrary to the behavior of some Pluronics that apparently exhibit interface stacking behavior and thus avoid forming self aggregates [45,46], Pluronic F68 has been shown to form self-aggregate structures, with reported CMC values varying widely (~0.04–1.14 mM) [47–49].

The potential for heterogeneous structures is made apparent by calculating the amount of excess phospholipid and cosurfactant available [41]. For simplicity, the emulsion formulation of the present study is assumed to consist of uniform droplets of 110 nm diameter. The total oil droplet surface area of a 10% (v/v) oil-in-water emulsion of 200 ml volume is thus calculated as ~1100 m^2. Comparing this value with the estimated surface coverage area of the detergent molecules in the emulsion allows an estimate of excess detergent available for micelle or liposome formation. Assuming a surface coverage area of 72 $Å^2$ per phospholipid molecule [50], the total interface area that could potentially be covered by the adsorbed phospholipids is 2100 m^2, nearly twice the available droplet surface area. In the case of the cosurfactant Tween 80 (assuming a surface area of 6 nm^2) [51], there are enough molecules to cover 2939 m^2, about 2.5 times the droplet surface area. In contrast, assuming a molecular surface area of 16 nm^2 [33], Pluronic F68 molecules could cover a total of 200 m^2, about five times less than the total emulsion droplet surface area. Therefore, at least for the phospholipid and Tween 80 molecules, it appears that they are present in excess and may be self-aggregating into liposomes, micelles, or mixed aggregate structures. This heterogeneity means that multiple interactions are probably occurring with resultant effects on mechanisms of stability and biological activity.

4.5. Biological activity

Of course, emulsion stability and structural properties are only precursors to the desired end goal of in vivo adjuvant activity. Determining if different formulation component sources affect the efficacy of the adjuvant formulation in eliciting an immune response is essential. To this end, adjuvant formulations containing shark or olive squalene and egg or soy PC were combined with a protein antigen for leishmaniasis and evaluated in a mouse model for immune response (Fig. 5). All of the formulations tested showed significant adjuvanticity in vivo, as more than 25 times higher IgG1 and IgG2a titers were detected in mice injected with antigen plus any emulsion formulation at both days 7 and 28 compared with injection of antigen alone. In general, no significant difference in immune response as determined by IgG1 and IgG2 assays was observed between the formulations. Thus, it appears that animal-source oil and PC components may be substituted by plant-source components without loss of adjuvant effect. Ongoing experiments in our lab are likewise testing the in vitro and in vivo efficacy of different emulsion component combinations, including the effects of Tween 80 vs. Pluronic F68 as cosurfactants.

Finally, in order to understand fundamental emulsion mechanisms of stability and biological activity, it is imperative that more extensive biophysical characterization is undertaken [37]. This will facilitate the development of more homogeneous formulations as well as allow the correlation of emulsion structure with stability and perhaps biological mechanisms of action. Empirical formulation methods will thus be replaced by systematic and rational design. To this end, future studies in our lab are being designed to employ extensive bioanalytical characterization techniques to monitor emulsion composition and interactions.

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