Comparison of Fluorinated and Nonfluorinated Lipids in Self-Adjuvanting Delivery Systems for Peptide-Based Vaccines

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Supporting Information

ABSTRACT: Safe immunostimulants (adjuvants) are essential for the development of highly potent peptide-based vaccines. This study examined for the first time whether fluorinated lipids could stimulate humoral immunity in vivo when conjugated to peptide antigen. The impact of fluorination on humoral immunity was tested using a library of peptide-based vaccine candidates against the group A streptococcus (GAS). The fluorinated constructs stimulated similar mouse IgG titers to those elicited by complete Freund's adjuvant (CFA) and were higher than those produced in mice that received the nonfluorinated constructs.

KEYWORDS: Fluorinated lipid, self-adjuvant, cellular immunity, humoral immunity, peptide-based vaccine, nanoparticle

Vaccination is one of the most successful tools for reducing the incidence of infectious diseases; exposing a person to live attenuated or inactivated pathogens can train the immune system to recognize a pathogen without falling ill. Although an inactivated pathogen is highly immunogenic, their use may cause allergies and autoimmune responses. Whole organism-based vaccines can have high batch-to-batch variation and often have poor stability (resulting in poor shelf life), thus they require cold chain (storage and transportation at fridge temperature). Subunit-based vaccines provide an opportunity to overcome these problems. Peptide subunit-based vaccines are not only more stable but also contain only the minimal immunogenic region of an antigen necessary to elicit the desired immune response. Therefore, only safe and effective epitopes can be selected, removing the risk of allergic and autoimmune responses. In contrast, classical vaccines always possess a large number of redundant components, which are not required for stimulation of protective immunity, yet they may cause an adverse effect.¹ However, peptides do not contain the danger signals that are needed to activate the immune system. Thus, an adjuvant/delivery system must be included to stimulate an immune response against the peptide antigen.²

The long-term pathogen-specific adaptive immune responses are generated through the propagation of memory B- and T-cells. Lymphocyte B-cells can produce pathogen-specific opsonic or neutralizing antibodies. Prophylactic vaccination aims to provide a protective immunity by the stimulation of B-cells.^{3,4} The vaccine also has to be recognized by antigen-presenting cells (APCs) to prompt an adaptive immune response. Multiple delivery systems have been reported to deliver peptide epitopes to the APCs, including lipopeptides, polymers, virus-like particles, and liposomes.^{5,6} However, fluorinated amphiphiles have not yet been examined for this purpose. These compounds have biomedical applications as components of liposomal drug carriers, for ultrasound-mediated delivery of lipophilic drugs or for two-dimensional crystallization of histidine (His)-tagged membrane protein.^{7–10} Fluorinated amphiphiles can form a third phase that is immiscible with aqueous and nonaqueous phases and thus self-assemble into nanoparticles.¹¹ They also do not accumulate in lipid membranes.¹² Fluorinated surfactants have a weak

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affinity for membrane lipids, which is advantageous because, unlike their nonfluorinated counterparts, they do not strip away the membrane lipids.¹³ Therefore, they are less hemolytic than hydrocarbon amphiphiles. These properties suggest that fluorinated lipids can serve as a nontoxic delivery system to develop peptide-based vaccines that self-assemble into nanoparticles.

Here, we designed and synthesized novel fluorinated lipo-alkynes **1** and **2** and their nonfluorinated analogues **3** and **4**, respectively (Figure 1). Compounds **1**–**4** were conjugated with

Figure 1. Fluorinated lipo-alkynes **1**–**2** and nonfluorinated lipo-alkynes **3**–**4**.

a peptide epitope using a copper-catalyzed alkyne–azide 1,3-dipolar cycloaddition (CuAAC) reaction. The resulting conjugates were assayed *in vivo* for their ability to elicit humoral immune responses in mice.

This work aimed to test whether fluorinated lipids formed particles that triggered humoral immunity when conjugated to B-cell epitope. For this purpose, fluorinated and nonfluorinated lipids (Figure 1) were conjugated to B-cell epitope derived from the M protein of group A streptococcus (GAS). Fluorinated lipids **1**–**2** were designed based on common features found in many peptide vaccine delivery systems that incorporate two lipidic chains (e.g., Pam2Cys (dipalmitoyl-*S*-glycerol cysteine),¹⁴ lipoamino acids,¹⁵ and dipalmitoyl serine (DPS)¹⁶). In contrast to these previously reported moieties, the new fluorinated lipids **1**–**2** have (1) stable ether bonds instead of the hydrolyzable ester linkage found, for example, in Pam2Cys, (2) no chiral center and therefore exists as a single isomer, (3) oxygen atoms in the hydrocarbon chain to enhance the solubility of the final compounds and (4) an alkyne moiety, allowing easy conjugation through CuAAC reaction. Fluorinated lipo-alkynes **1**–**2** were synthesized in three steps (Scheme 1). First, the fluorinated diols **5** and **6** were treated with an alkyl halide (*n*-butyl bromide or *n*-octyl bromide) to afford the fluorinated alcohols **7** and **8** in 53% and 43%, respectively. This substitution reaction was achieved in the presence of sodium hydride (NaH) that acted as a base. The phase transfer catalyst tetrabutylammonium iodide (TBAI) was used to convert the alkyl bromide into the more reactive iodo-

Scheme 1. Synthesis of Fluorinated Lipo-alkynes **1**–**2**

analogue *in situ*. Dimethylformamide (DMF) was used as a polar aprotic solvent. We previously found that the alkoxide salt of fluorinated alcohol had limited solubility at room temperature and that NaH had a destructive effect on the fluorinated compounds by detaching fluorine atoms at high temperature.⁸ These problems were overcome by sonicating the reaction mixture for 2 h at room temperature. In the next step, a solvent-free method was used: fluorinated alcohols **7** or **8** were subjected to prolonged sonication with epichlorohydrin, tetrabutylammonium bromide (TBABr, a phase transfer catalyst), and fine powdered NaOH to afford **9** and **10** in 56% and 33% yield, respectively. The attempt to prepare the alkyne derivatives **1** and **2** by reacting diglyceride alcohols **9** or **10** with propargyl bromide and NaH using DMF as a solvent was unsuccessful, most likely due to the detachment of fluorine atoms from the fluorocarbon backbone resulting from the fluorinated compound's prolonged exposure to NaH in DMF. The fluorinated lipo-alkynes **1**–**2** were successfully obtained in 30% and 50%, respectively, when the tetrahydrofuran (THF, a less polar solvent) was used. The lipo-alkynes **3**–**4** (Figure 1) were synthesized as previously described.¹⁷

GAS is a Gram-positive bacteria that colonizes the throat of the host and causes pharyngitis.¹⁸ Inadequate early treatment of GAS infections can result in postinfectious autoimmune complications including rheumatic fever and rheumatic heart disease, resulting in a half a million deaths worldwide per year.¹⁹ Whole cell-based vaccines may also cause these autoimmune sequelae, thus only subunit vaccines are considered safe for developing a GAS vaccine.²⁰ Peptide-based vaccines are among the most intensively studied for this purpose and epitopes from the major virulent factor of GAS (M-protein) are one of the most promising antigens. B-cell epitope J14 (KQAEDKVKASREAKKQVEKALEQLEDKVK), derived from the conserved C-repeat region of the M protein, has been chosen as a model antigen for evaluating the ability of fluorinated lipo-peptides to stimulate humoral immunity in this study. This epitope has been previously evaluated in combination with a variety of delivery systems and antibody produced against this epitope has been shown to be opsonic against clinical GAS isolates.^{21–24} J14 azide (**11**) was

synthesized using Fmoc-SPPS as previously described.⁵ The final vaccine candidates **12–15** were obtained using the CuAAC reaction in the presence of copper wire at 50 °C. HPLC was used to monitor the progress of the reaction and **12–15** were obtained in 42–65% yields after purification and freeze-drying (Scheme 2).

Scheme 2. Synthesis of Vaccine Candidates **12–15**

Homogenous solutions of the compounds in PBS were prepared by sonication and vortexing. Particle sizes of compounds **12–15** were measured by using dynamic light scattering (DLS) and transmission electron microscopy (TEM) (Figure 2). Compounds **12–15** self-assembled into 10–15 nm particles in PBS.

The α -helical secondary conformation of the J14 peptide, derived from GAS M protein, is crucial for antibody recognition.²⁵ The secondary structure of the vaccine candidates **12–15** was measured using the circular dichroism (CD) analysis. Minima at approximately 205 and 222 nm correlate with the formation of α -helices (Figure 2e). All compounds showed a similar predisposition to adopting an α helical conformation.

The toxicity of the fluorinated compounds **12** and **13** was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and hemolytic assays. The non-fluorinated analogue **14** was used as a reference. None of the compounds demonstrated measurable cytotoxicity against Caco-2 cells nor caused lysis of human red blood cells (Figure 3).

The ability of vaccine candidates **12–15** to elicit a humoral response against GAS was investigated in inbred B10.BR mice. Mice (5 mice/group) were immunized subcutaneously at the tail base on day 0 with 30 μ g of vaccine candidates **12–15**. The positive control group was treated with J14 emulsified in

Figure 2. Shape and particle size of compounds (a) **12**, (b) **13**, (c) **14**, and (d) **15** was measured using transmission electron microscopy (TEM). All four compounds showed 10–15 nm spherical-shaped particles (bar 200 nm). (e) CD spectrum of vaccine candidates **12–15**.

complete Freund's adjuvant (CFA). The negative control group was administered with sterile filtered PBS. An additional two boosts of each immunogen were introduced at days 21 and 28 after the primary immunization. Sera samples were collected prior to each boost and 9 days after the final boost, and the levels of J14-specific immunoglobulin G (IgG) were determined using ELISA (Figure 4). Interestingly, fluorinated derivatives **12** and **13** induced higher antibody titers than nonfluorinated analogues **14–15** and were very similar to those induced by the positive control J14+CFA.

The availability of nontoxic and efficient adjuvants that can stimulate cellular and/or humoral immunity without causing side effects is very limited. Peptide alone are unable to stimulate the required immune response; thus, there is increased demand for the discovery of new molecules that can play this crucial adjuvanting role. Here, we designed and synthesized fluorinated and nonfluorinated lipoalkynes **1–2** and **3–4**, respectively (Figure 1 and Scheme 1). The stimulation of humoral immunity was tested using fluorinated (**12–13**) and non-fluorinated (**14–15**) compounds that carried the B-cell GAS epitope, J14 (Figure 4). The fluorinated compounds **12–13**, which contained the highly hydrophilic J14 peptide, had the same particle size as the nonfluorinated analogues **14–15** (10–15 nm). Despite the similarity in particle size of all tested compounds (10–15 nm), the fluorinated derivatives **12–13** showed higher IgG titers than their hydrogenated analogues **14–15**. The fluorinated derivatives were more hydrophobic,

Figure 3. Toxicity evaluation of compounds 12, 13, and 14 in (a) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: Caco-2 cell viability after 4 h exposure to compounds 12, 13, and 14 at concentrations of 50, 100, and 200 μ M. (b) Hemolysis caused by compounds 12, 13, and 14 (at 50, 100, and 200 μ M). The percentage hemolysis is calculated by assuming that the positive control SDS will give 100% hemolysis. Error bars indicate two independent assays conducted; each assay was performed in triplicate.

Figure 4. J14-specific IgG titers elicited in response to vaccination of B10.BR mice ($n = 5$ /group) at day 37, serum IgG antibody titer (log 10). Mice received a primary subcutaneous immunization on day 0, followed by two boosts on days 21 and 28. Samples were collected on day 37. Statistical analysis was performed by one-way ANOVA followed by the Tukey's multiple comparisons test (ns > 0.05, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001). Statistical differences below the line are the comparison between all of the groups versus PBS, while the statistical differences above the line are the comparison between J14+CFA versus 12, 13, 14, and 15 groups.

thus may experience enhanced up-take by APCs through the endocytosis pathway. Consequently, the antigen would be processed and presented by MHC class II more efficiently, resulting in a stronger humoral immune response. However, these differences were not statistically significant.

In conclusion, four vaccine candidates were synthesized through conjugation of lipids to a highly hydrophilic B-cell epitope, J14. The produced vaccine candidates 12–15 formed small nanoparticles, 10–15 nm. The two lead fluorinated vaccine candidates 12–13 induced a higher level of IgG titers than nonfluorinated analogues 14–15. Thus, we have demonstrated that fluorinated lipids conjugated to peptide antigen can induce effective immune response once they form nanoparticles. These fluorinated analogues could be attractive and safe alternatives to traditionally used lipids used in peptide-based vaccine delivery.

EXPERIMENTAL PROCEDURES

Peptides J14,⁵ 11,⁵ and compounds 3–4 were synthesized as reported.¹⁷

General Method for the Synthesis of Vaccine Candidates 12–15. A mixture of 11 (1 equiv) and lipoalkynes 1, 2, 3, or 4 (1.5 equiv) was dissolved in DMF (1 mL). Copper wire (80 mg) was added to the mixture. The reaction mixture was degassed for 30 s by nitrogen bubbling and stirred at 50 $^{\circ}$ C under a nitrogen atmosphere. The progress of the reaction was monitored by using analytical HPLC and ESI-MS until 11 was completely consumed.

Vaccine Candidate 12. The reaction mixture was purified by using semipreparative HPLC C-4 column (30–70% solvent B from 15 to 75 min). Analytical HPLC analysis (C-4 column) $t_R = 30.4$, purity > 95%. Yield: 42%. ESI-MS: m/z 1523.7 (calc 1523.5) $[M + 3H]^{3+}$; 1146.2 (calc 1142.9) $[M + 4H]^{4+}$; 914.3 (calc 914.1) $[M + 5H]^{5+}$; 762.4 (calc 762.3) $[M + 6H]^{6+}$; MW 4567.5.

Vaccine Candidate 13. The reaction mixture was purified by using semipreparative HPLC C-4 column (30–70% solvent B from 15 to 75 min). Analytical HPLC analysis (C-4 column) $t_R = 25.6$, purity > 95%. Yield: 43%. ESI-MS: m/z 1427.8 (calc 1427.5) $[M + 3H]^{3+}$; 1071.0 (calc 1070.9) $[M + 4H]^{4+}$; 856.9 (calc 856.9) $[M + 5H]^{5+}$; 714.6 (calc 714.3) $[M + 6H]^{6+}$; MW 4279.7.

Vaccine Candidate 14. The reaction mixture was purified by using preparative HPLC C-4 column (40–60% solvent B from 10 to 30 min). Analytical HPLC analysis (C-4 column) $t_R = 28.4$, purity > 95%. Yield: 65%. ESI-MS: m/z 1331.5 (calc 1331.6) $[M + 3H]^{3+}$; 999.3 (calc 998.9) $[M + 4H]^{4+}$; 799.3 (calc 799.3) $[M + 5H]^{5+}$; 666.3 (calc 666.3) $[M + 6H]^{6+}$; MW 3991.74.

Vaccine Candidate 15. The reaction mixture was purified by using preparative HPLC C-4 column (30–70% solvent B from 15 to 75 min). Analytical HPLC analysis (C-4 column) $t_R = 28.2$, purity > 95%. Yield: 62%. ESI-MS: m/z 1331.9 (calc 1331.6) $[M + 3H]^{3+}$; 999.3 (calc 998.9) $[M + 4H]^{4+}$; 799.4 (calc 799.3) $[M + 5H]^{5+}$; 666.3 (calc 666.3) $[M + 6H]^{6+}$; MW 3991.74.

Subcutaneous Immunization of Mice for Humoral Immunity. Female inbred B10.BR mice (6–8 weeks) were used in this study (Animal Resource Centre, Western Australia). All animal protocols used were approved by the Animal Ethics Committee (The University of Queensland), in accordance with National Health and Medical Research Council (NHMRC) of Australia guidelines.

Mice (5 per group) were subcutaneously immunized at the tail base with 30 μ g of vaccine candidates 12–15 dissolved in 50 μ L of sterile PBS (Griffith University, Gold Coast, Australia). Similarly, a negative control group was administered 50 μ L of PBS and the positive control received 30 μ g of J14+CFA (total volume of 50 μ L). Boosts of the same dosage were given on days 21 and 28 after primary immunization.

Serum Collection. Final serum was collected on day 39. Blood (10 μ L) was collected from the tail artery of each mouse and diluted 10 times with PBS. The serum was extracted by centrifugation at 1500g for 10 min and stored at -20° C.

Determination of Antibody Titers. The ability of the serum IgG antibodies to detect the J14 epitope was tested using ELISA.⁵ Briefly, ELISA plates were coated with J14 (10 mg/mL) in carbonate coating buffer overnight at 4 °C and blocked with 150 μ L/well of 5% skim milk for 90 min at 37 °C. Serial dilutions of collected sera were prepared in 0.5% skim milk/PBS-Tween-20 buffer, starting at a concentration of 1:100, followed by 1:2 dilutions. Absorbance values were read at 450 nm in a microplate reader after the addition of secondary antibody (peroxidase-conjugated antimouse IgG) and O-phenylenediamine. The antibody titer was identified as the lowest dilution that gave an absorbance more than three standard deviations above the mean absorbance of control wells (which contained normal mouse serum). Statistical analysis ($p < 0.05$ = statistical significance) was performed with one-way ANOVA followed by Tukey's post hoc test.

MTT Cytotoxicity Assay. The experiment was performed as reported.²⁶ Caco-2 cells were plated at 2.5×10^4 cells/well onto 96-well plates. The cells were used after 5 days and the cell medium was replaced with the test compounds in triplicate at a concentration of 50, 100, and 200 μ M (in PBS) and incubated for 4 h. The solutions were removed and 20 μ L of MTT (5 mg/mL in PBS) with 50 μ L of cell medium was added to each well. After another 4 h of incubation, the MTT medium was removed and formazan crystals were solubilized with 50 μ L of DMSO. The SpectraMax 250 microplate reader was used to measure the absorbance of each well at 550 nm. The percentage of cell viability for each sample was calculated by comparing with 100% viability samples (PBS as positive control) and 0% viability samples (SDS). The experiment was performed as three independent replicates.

Hemolytic Assay. The experiment was performed as previously reported (protocol approved by the University of Queensland Ethics Committee, approval number 2009000661).²⁶ Briefly, a solution of the tested compounds (50, 100, and 200 μ M in PBS) was added in 100 μ L triplicates to human erythrocytes (from healthy volunteers) and incubated at 37 °C for 1 h. After 1 h incubation, the plates were centrifuged at 750g for 15 min to remove intact erythrocytes and 75 μ L of supernatant was drawn and transferred to a new plate for UV measurement. The absorbance of each sample was recorded at 540 nm using a Spectramax 250 microplate reader. Sodium dodecyl sulfate (SDS) 100 μ g/mL was used as a positive control, and PBS was used as the negative control. The equation below was used to calculate the percentage of hemolysis caused by each test.

$$\text{hemolysis(\%)} = \frac{A_{540} - A_{540,\text{min}}}{A_{540,\text{max}} - A_{540,\text{min}}}$$

where A_{540} was the absorbance of the test sample at 540 nm, $A_{540,\text{min}}$ was the absorbance of the negative control, and $A_{540,\text{max}}$ was the absorbance of the positive control.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.6b00453.

Additional information includes toxicity evaluation of compounds, synthetic procedure for compounds 1, 2, and 7–10, the ¹H and ¹³C NMR spectra of new compounds, and the HPLC and mass spectra of 12–15 (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

APCs, antigen-presenting cells; CuAAC, copper (wire)-catalyzed alkyne–azide cycloaddition; TBABr, tetrabutylammonium bromide; TBAI, tetrabutylammonium iodide

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