SUPPORTING INFORMATION

Formulation and Characterization of Nanoemulsion Intranasal Adjuvants: Effects of Surfactant Composition on Mucoadhesion and Immunogenicity

Pamela T. Wong,[1] Su He Wang,[1] Susan Ciotti,[2] Paul E. Makidon,[1] Douglas M. Smith,[1] Yongyi Fan,[1] Charles F. Schuler IV,[1] James R. Baker Jr.[1,2]*

[1]Michigan Nanotechnology Institute for Medicine and Biological Sciences and Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan, [2]NanoBio Corporation, Ann Arbor, Michigan

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Figure S1. Chemical structures of some surfactants used in NEs: (A) CPC, (B) DODAC, (C) CAB, (D) SDS, (E) Tween80, (F) P407.

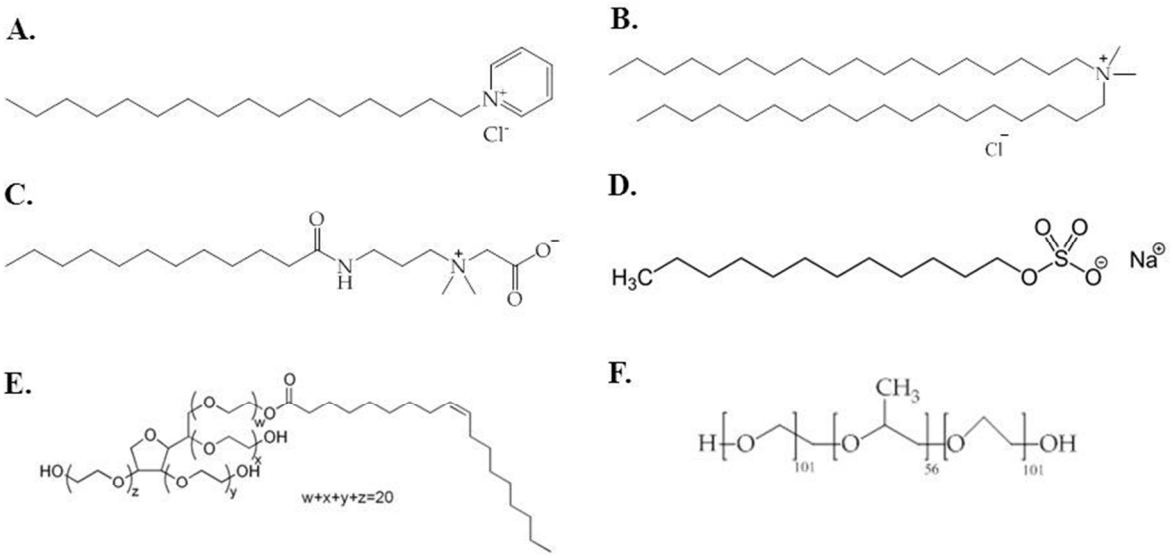
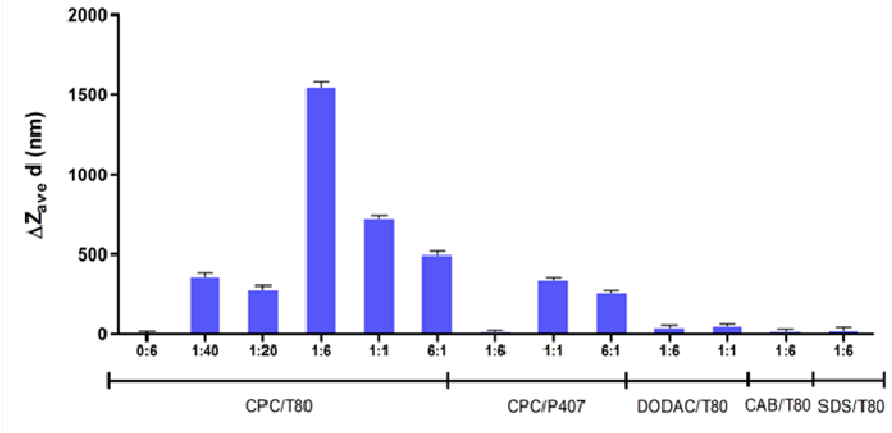
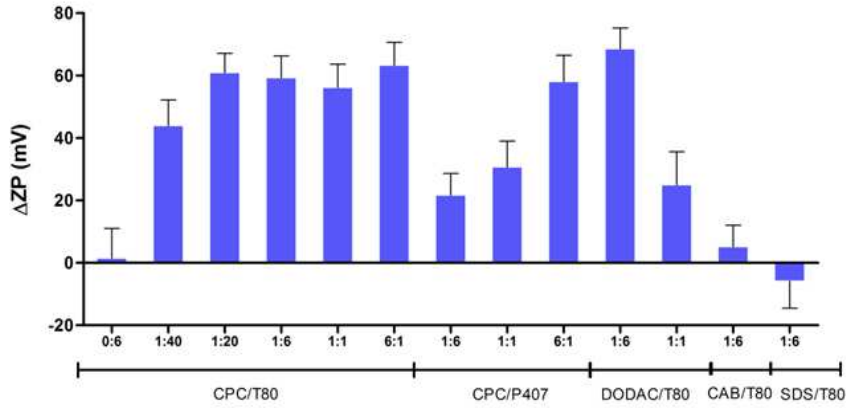


Figure S2. (A) ΔZ_{ave} values ($Z_{ave\ final} - Z_{ave\ init}$) and (B) ΔZP values ($ZP_{init} - ZP_{final}$) for the representative NE formulations described in Figure 2 upon mucin addition. 0.1% NE was incubated with 0.05 mg/mL mucin in 1 mM HEPES pH 7. Error bars for Z_{ave} measurements represent standard deviation as calculated from PdI values, or zeta deviation for ZP measurements.

(A)



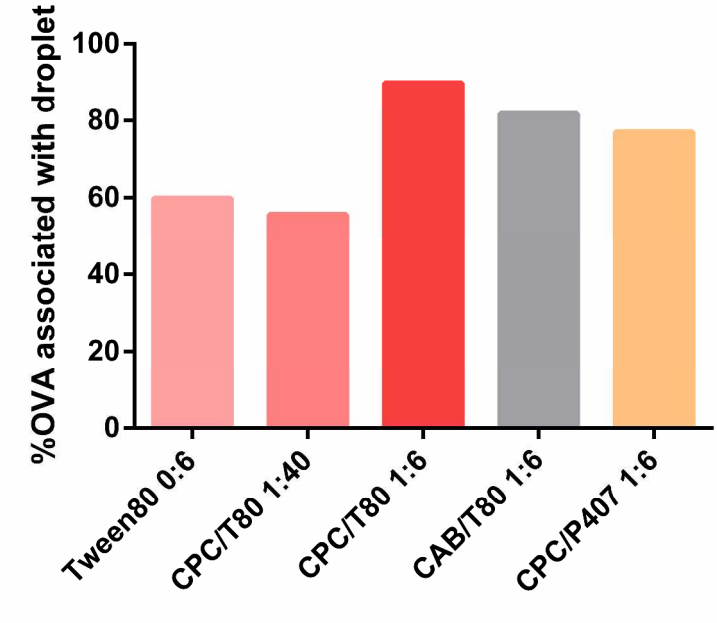
(B)



Antigen Incorporation:

Antigen incorporation on the NE droplet was determined by separating NE droplets from aqueous solution through ultracentrifugation. Briefly, a 2 mL solution of 20% NE (Tween80 0:6, CPC/Tween80 1:40 or 1:6, CPC/P407 1:6, or CAB/Tween80 1:6) with 100 μg/mL OVA was prepared in PBS, and ultracentrifuged in a Type 100 Ti rotor at 100,000 x g for 2 h. The aqueous phase was removed from the bottom of the tube with a syringe, and the OVA concentration was determined by HPLC analysis. (Figure S3). We found that most (>50%) of the OVA is incorporated on the droplet of Tween80 0:6, and CPC/Tween80 1:40 and 1:6, suggesting that the NE-OVA interaction is not solely electrostatic, as lack of CPC did not prevent incorporation.

Figure S3. % OVA incorporation on the NE droplet for 20% NE incubated with 100 μg/mL OVA in PBS. NE droplets were separated from the aqueous phase through ultracentrifugation and OVA content in the aqueous phase was determined by HPLC analysis.



To determine whether antigen incorporation on the NE alters the droplet properties, 20% NE (Tween80 0:6, CPC/Tween80 1:40 or 1:6, CPC/P407 1:6) was mixed with 1 mg/mL ovalbumin antigen (OVA) in PBS, similar to the vaccination mixture used *in vivo*. OVA association did not significantly alter the NE PS when measured in PBS (not shown). To keep measurement conditions for mucin binding consistent, particle size and ZP were measured by diluting NE-OVA to 0.1% in 1 mM HEPES pH 7 (+/- 0.05 mg/mL mucin). OVA did not markedly decrease the ZP of Tween80 0:6, or CPC/P407 1:6, (Table S1). However, OVA decreased the ZP of CPC/Tween80 1:40 from 12.4±6.3 mV to -0.9±0.1 mV, and that of CPC/Tween80 1:6 from 46.1 ± 5.6 mV to 14.8±4.5 mV, by neutralizing the cationic surface with its negative charge. NEs with higher ZP_{init} thus retained more charge upon OVA incorporation than those with lower charge.

While ΔZ_{ave} and ΔZP with mucin were smaller for NE-OVA compared to NE alone (no OVA), the general trends observed were similar. CPC/Tween80 1:6-OVA and CPC/P407 1:6-OVA had final ZP values upon mucin association similar to the NEs without OVA, reflecting mucin association despite the lowered ZP_{init} (Table S1). Interestingly, because the ZP_{init} of CPC/Tween80 1:40-OVA was reduced to near neutral, no significant ΔZP with mucin was observed, (ΔZP = 30 mV NE without OVA), reflecting no association. OVA did not alter the ΔZP of Tween80 0:6. Thus the trend in ΔZP magnitude observed for NE alone was maintained for NE-OVA (Tween80 0:6<CPC/Tween80 1:40<CPC/Tween80 1:6). ΔZ_{ave} magnitudes for NE-OVA were also reduced for all NEs, however the trend seen for the NEs without OVA was maintained (Tween80 0:6<CPC/Tween80 1:40<CPC/Tween80 1:6) (Table S1). Thus, while antigen incorporation slightly alters the mucoadhesive properties of the emulsion, general trends observed for NE alone apply to NE-antigen as well. It may be important to determine the

optimal concentration ranges for different protein antigens used with the NE system that would allow maximal antigen dosage yet maintain enough droplet charge for mucoadhesion.

Table S1: Effects of OVA incorporation on NE properties and mucin association for Tween80 0:6, CPC/Tween80 1:40, 1:6, and CPC/P407 1:6. 20% NE was incubated with 1 mg/mL OVA in PBS for 10 min as described above. NE alone or NE-OVA were then diluted to a final concentration of 0.1% NE (+/- 5 μg/mL OVA) with or without 0.05 mg/mL mucin in 1 mM HEPES pH 7. ZP was measured pre-mucin addition (ZP_{init}) or post-mucin addition (ZP_{final}) ($\Delta ZP = ZP_{init} - ZP_{final}$), and the change in particle size with mucin (ΔZ_{ave}) was also measured.

		ZPinit (mV)	ZPfinal (mV)	ΔZP (mV)	ΔZave (nm)
Tween80 0:6	-OVA	-14.5±4.15	-14.1±15.0	-0.4±6.1	-13.6±15.0
	+OVA	-12.2±4.9	-12.9±5.1	0.7±7.1	-18.9±14.7
CPC/Tween80 1:40	-OVA	12.4±6.3	-19.4±6.0	31.6±8.7	29±19.4
	+OVA	-0.9±0	-1.1±0.0	0.1±0.0	1.7±17.5
CPC/Tween80 1:6	-OVA	46.1±5.6	-3.9±3.6	50.0±6.6	1237.9±38.0
	+OVA	14.8±4.5	-5.5±3.8	20.3±5.9	461±50.4
CPC/P407 1:6	-OVA	23.8±5.0	26.5±7.0	-13.6±15.0	19.2±6.6
	+OVA	16.8±8.4	21.1±9.9	-13.6±15.0	9.6±5.8

Figure S4. Dose-dependent SPR sensorgrams of CPC/Tween80 (1:6) injected over (A) a mucin-immobilized carboxymethyl cellulose (mucin/CMC) dextran surface and (B) a CMC dextran reference surface. NE dilutions were prepared in 1 mM HEPES, pH 7 (buffer 1). Increasing the ionic strength of the buffer eliminated binding to both the mucin/CMC and CMC only channels. (C) CPC/Tween80 (1:6) injected over the same mucin/CMC surface and (D) the CMC dextran surface. NE dilutions were prepared in 10 mM HEPES, 100 mM NaCl, 0.005% Tween 20, pH 7 (buffer 2). The concentrations of NEs injected are indicated. Negative RUs observed in (C) and (D) are attributable to changes in the refractive index due to the injection of NE, and reflect no association to the chip.

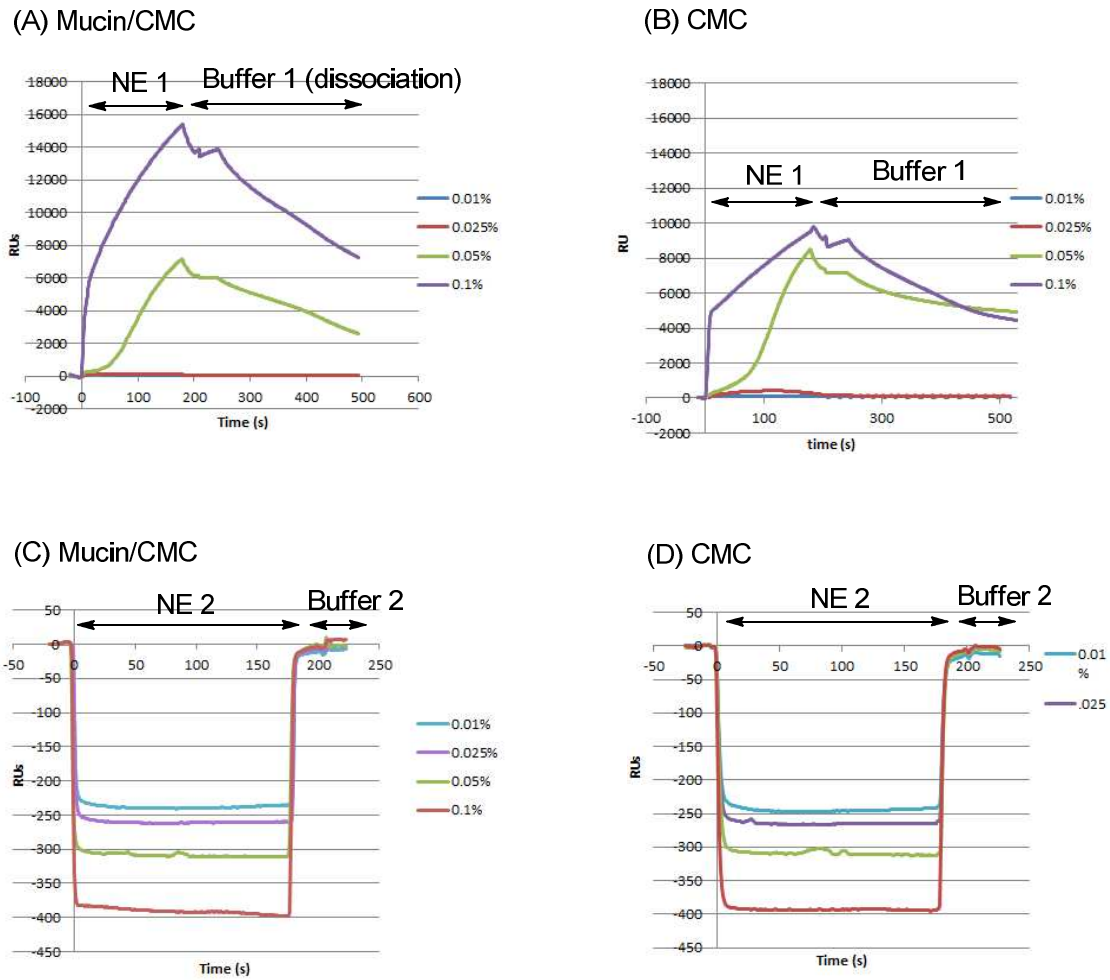
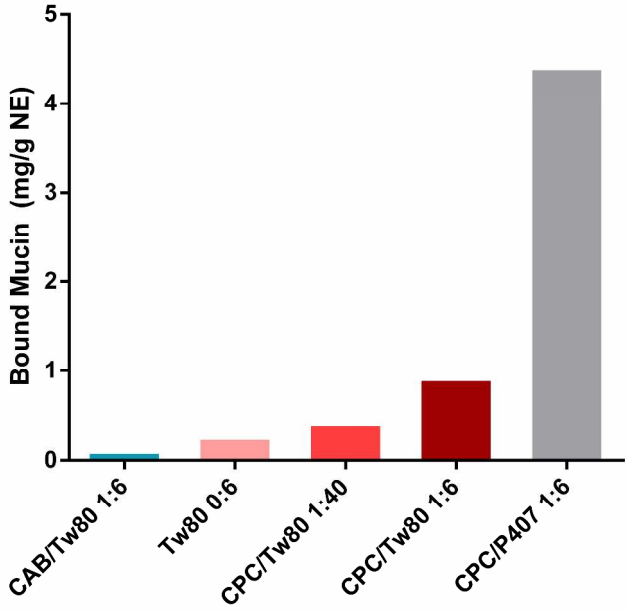
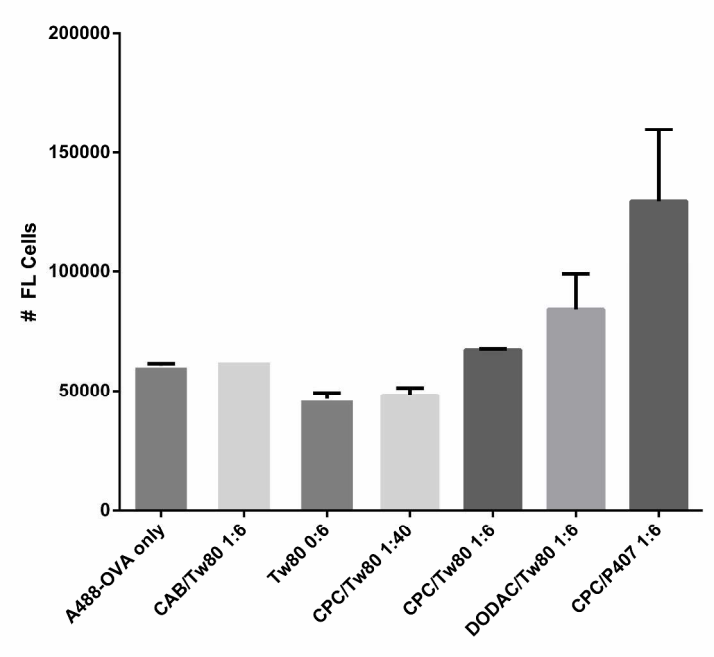


Figure S5. The amount of mucin adsorbed to the NE droplets for different representative formulations were evaluated. NE oil droplets were separated from the aqueous solution through ultracentrifugation. 10% NE was incubated with 0.5 mg/mL mucin in PBS, pH 7.4, and ultracentrifuged in a Type 100 Ti rotor at 100,000 x g for 1 h at RT. The aqueous solution was isolated using a syringe, and the amount of mucin was determined by measuring the absorbance at 280 nm.



The amount of mucin bound to the NE droplet corresponded with particle size and zeta potential measurements, as well as with SPR analysis. For example, CPC/Tween80 1:6, which showed a large increase in particle size and large drop in zeta potential with mucin had 3.6 and 8.9 times the amount of bound mucin than Tween80 0:6 and CAB/Tween80 1:6 formulations, respectively, which both showed minimal changes in both particle size and zeta potential with mucin.

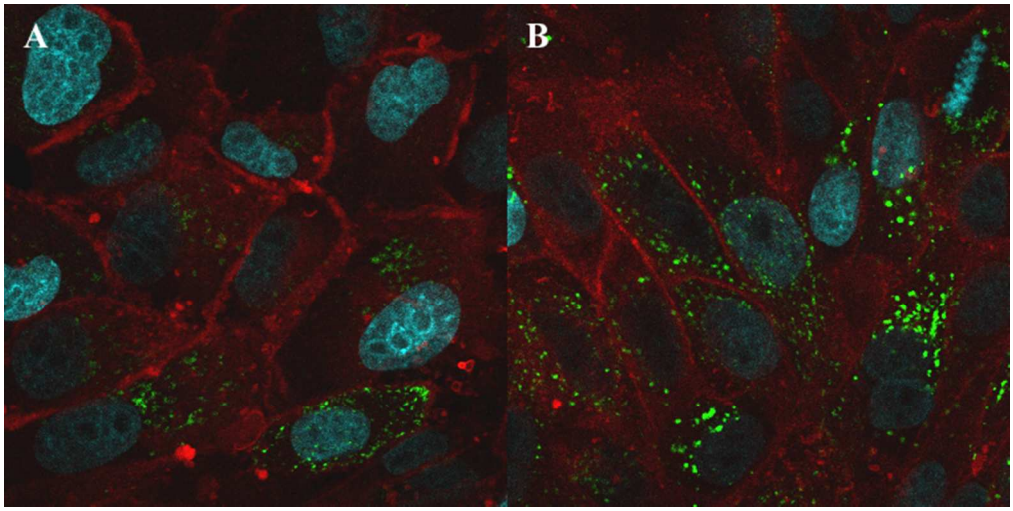
Figure S6. Flow cytometry analysis of RPMI 2650 cells treated with 0.1% NE containing 1 μg/mL A488-OVA for 1.5 h at 37°C. 5 x 10^5 RPMI 2650 cells/well were seeded on a 24 well plate O/N, and then incubated with NE-OVA for 1.5 h at 37°C . Cells were trypsinized, and washed 2x in PBS containing 0.1% BSA, 0.1% NaN_3. Flow analysis was performed on an Accuri C6 flow cytometer (BD Biosciences) and 100,000 events were collected. Fluorescent cells were defined as those cells with fluorescence greater than that of untreated cells. Error bars represent standard deviation of duplicate measurements.



While confocal is a more sensitive method for detecting the differences in antigen uptake and localization, flow analysis confirmed the results of our confocal studies. CPC/Tween80 1:6, DODAC/Tween80 1:6 and CPC/P407 1:6 all enhanced the uptake of OVA in epithelial cells relative to antigen alone and CAB/Tween80 1:6, Tween80 0:6 and CPC/Tween80 1:40, as reflected by the increase in the number of fluorescent cells. This is consistent with Figure 6II. CPC/P407 1:6 (129,732 ± 30,734 cells) enhanced uptake in the greatest number of cells, more than doubling the number of fluorescent cells compared to antigen alone (60,702 ± 849 cells). This was

followed by DODAC/Tween80 1:6 (84,190 ± 14,810 cells). These results confirm Figure 6II (E&F). With flow cytometry, however, differences in cellular distribution of fluorescence, such as between CPC/Tween80 1:6 which produced intense puncta of fluorescence in some cells and DODAC/Tween80 1:6 which showed more uniform uptake cannot be distinguished. Thus both confocal and flow methods of analysis measuring uptake are important for analysis of uptake. While CPC/Tween80 1:40 showed slightly more uptake by confocal than A488-OVA only, and CAB/Tween80 1:6 treated cells, it showed slightly less by flow analysis. This could be due to the differences in cell preparation for the two techniques, as cells used in the flow studies were not fixed and were trypsinized after NE treatment prior to analysis, whereas cells used for confocal were fixed and not trypsinized prior to imaging. Furthermore, CPC/Tween80 1:40 shows punctate areas of intense fluorescence in a very low number of cells by confocal, which may be harder to detect by flow cytometry.

Figure S7. Confocal fluorescence microscopy of RPMI 2650 cells treated with (A) 0.05% CPC/Tween80 1:6, or (B) 0.05% DODAC/Tween80 1:6 containing 20 mg/mL A488-OVA (green fluorescence) for 2 hr at 37°C. The cell membrane was then stained with Cell Mask Orange© (Life Technologies, Carlsbad, CA) (red fluorescence) for 5 min before fixation in paraformaldehyde. Nuclei were stained with DAPI (blue fluorescence).



Under these treatment conditions at 37°C, the majority of the OVA was found within the confines of the cellular membrane, confirming internalization of the antigen as opposed to simply adherence of the NE-OVA to the cell surface.