Title: Zwitterionic Surfactant as a Promising Non-Cytotoxic Carrier for Nanoemulsion-Based Vaccine Development

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Zwitterionic Surfactant as a Promising Non-Cytotoxic Carriers for Nanoemulsion-Based Vaccine Development

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Abstract: Motivated by the lack of noncytotoxic carriers in the current vaccine, we pursued the possibility of using a zwitterionic surfactant as a carrier or co-carrier in combination with other known adjuvants to improve their delivery efficiency with antigen for a nanoemulsion-based vaccine. We identified that a nanoemulsion formulation consists of a specific zwitterionic surfactant can effectively mediate cellular uptake of antigen despite not having cytotoxicity as compared to a nanoemulsion consists of a cationic surfactant. We report here the first study of a zwitterionic surfactant that consists of a positive charge in the outer layer of the polar head group and a hydrophobic tail is a promising approach for enhancing the carrier's efficacy with no noticeable toxicity under experimental condition. However, zwitterionic surfactant that has positive charge in the outer layer with additional hydrophobicity due to the presence of aromatic ring had minimal cellular uptake and transfection efficacy.

The mucosal route of vaccination has benefits over intramuscular (IM) and subcutaneous administration. One benefit is its potential to induce mucosal immunity against respiratory and gastrointestinal infections at the point of the pathogens entry.[1] It has also been shown that mucosal immunization can cause protective immunity at distant mucosal surfaces.[2] Given this, the nasal cavity is a leading site for mucosal vaccination due to its accessibility and its moderately permeable epithelium which permits access to immune-reactive sites.[3],[4] Unfortunately, safe and effective carriers for nanoemulsion (NE) based vaccines have been difficult to identify.

Nanoemulsion based carriers are promising candidates under development as nasal vaccine carriers. NE provides enhanced mucoadhesion leading to longer retention of antigens in the nasal mucosa. NEs facilitate antigen permeation across the mucous layer and enhance cellular uptake. NEs are nanometer scale (d=200–700 nm) oil-in-water emulsions consisting of a combination of nonionic and ionic surfactants, a co-solvent (ethanol), oil (soybean oil), and water.[5] Prior work by our group has produced a number of NE formulations by varying the combinations of nonionic and ionic surfactants, which were chosen based on their hydrophilic−lipophilic balance (HLB) values and differing polar head groups.[6] A typical NE formulation consists of the nonionic surfactant Tween80 and the cationic surfactant cetylpyridinium chloride (CPC). This formulation has demonstrated effective nanoemulsion based adjuvant activity for a variety of antigens and has induced systemic antibody titers comparable to injected aluminium-based vaccines.[7] Despite their utility, cationic surfactants including CPC usually are associated with cytotoxicity and also cations have short *in vivo* circulation half life.[8] While in some cases this is thought to be inherent to carrier activity, we sought to develop a compound that impart the effects of a cationic charge but keep it nontoxic by polarizing the molecule so that it could be used as an effective carrier or co-carrier along with other known adjuvants to enhance delivery efficacy with antigen.

This led us to explore nanoparticles with zwitterionic (ZI) surfaces. ZI particles have been shown to exhibit a long circulatory half-life,[8b] enhance enzyme activity,[9] and demonstrate low toxicity in cell-based assays.[10] Recently, a mannosylated ZI-based cationic liposome was designed as a DNA vaccine delivery system to promote immunogenicity with lower cytotoxicity.[11] Interestingly, ZI polysaccharides without subsequent modification were successfully used as carriers.[12] Inclusion of ZIs were found to be significantly advantageous in both studies.[11-12] Different study identified that subtle tuning of the head group charge orientation of ZIs resulted in significant alterations of their activities.[13] These reports suggest that ZIs with a positive outer layer showed superior antimicrobial properties than similar ZIs with negative outer layers.[13] Motivated by these reports, we hypothesize that ZIs could be substituted for cationic surfactants in biological applications with potentially less toxicity.

Our objective is twofold: First, we pursued the possibility that ZIs could be used as a nontoxic alternative to cationic surfactants in NE carriers for both DNA and protein-based vaccines. Second, we sought to evaluate the effect of changing the structure and charge orientations of ZIs on NE activity as a carrier. In this study, four ZI molecules were designed, synthesized and compared with a commercially available cationic surfactant that has been used previously as a carrier in NE formulations.[6] These small ZI molecules consist of different charge orientations (Figure **1**). Among the four ZI molecules, three have positive charges on the outer layer, while the fourth has a positive charge inside. We performed cytotoxicity, binding, transfection, and uptake studies with NE formulations (NEs **1**-**5**) containing these molecules (compounds **1**-**5**). The result showed NEs **1**-**5** significantly differed from each other in size, ζ-potential and cytotoxicity. Results also suggest that substituting the small molecule consisting of hydrophobic tails and ZI head with positive charges in the outer layer could improve the plasmid and ovalbumin antigen (OVA) binding capability, antigen uptake and transfection efficacy of NE without any noticeable cytotoxicity under experimental condition.

We designed and synthesized ZI surfactants that consist of either positive charges in the outer or inside layer of the hydrophilic head group. The overall length of hydrophobic chains in all synthesized compounds are similar to CPC, the cationic surfactant we wished to replace. Compounds **1**, **3** and **4** have similar head groups. However, head groups on compounds **1** and **4** have reversed charge orientations. Compound **3** consists of a positively charged group in the outer layer and an aromatic ring that increases the hydrophobicity of the head group compared to compounds **1** and **4**. Compounds **2** and **5** contain phosphorylcholine (ZI) and pyridine (cationic) head groups respectively while compound **2** (ZI) also has a positive charge in the outer layer.

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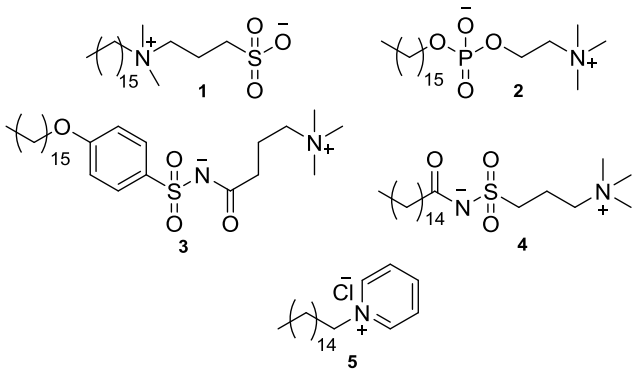
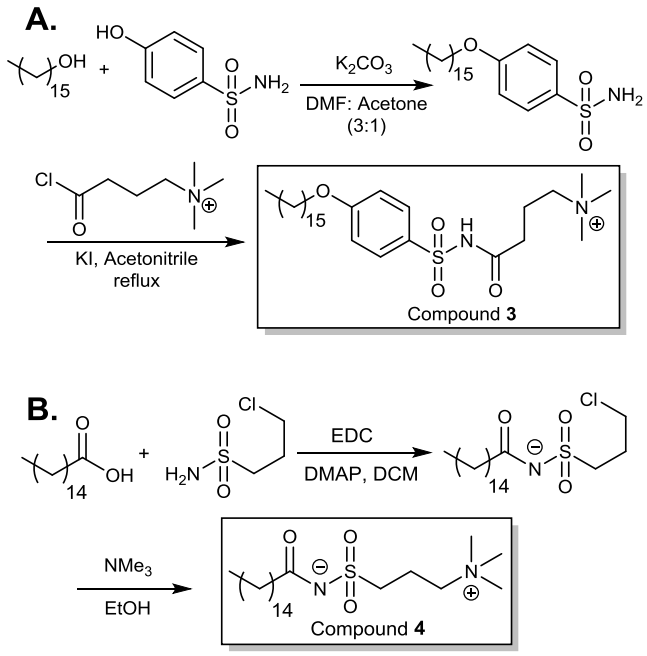


Figure 1. Compounds (**1**-**5**) used in (**1**-**5**) NE formulations.



Scheme 1. Synthetic route of compounds **A) 3** and **B) 4**.

Compounds **1** and **2** were synthesized as described previously.[14] Compound **3** was synthesized in two steps as shown in Scheme 1A. First, 4-(hexadecyloxy)benzenesulfonamide was synthesized by reacting 1-iodohexadecane and 4-hydroxybenzenesulfonamide in the presence of K_2CO_3. Subsequently, 4-(hexadecyloxy)benzenesulfonamide and (3-carboxypropyl)trimethylammonium chloride were refluxed in acetone in the presence of KI to yield compound **3**.[15] N-acylsulfonamides are typically synthesized in the presence of DCC and DMAP. However, compound **3** was not obtained under this condition. Compound **4** (Scheme 1B) was synthesized in two steps; we first synthesized ((3-chloropropyl)sulfonyl)(palmitoyl)amide by a coupling reaction between palmitic acid and 3-chloropropane-1-sulfonamide. Next, trimethyl amine substituted chloride of ((3-chloropropyl)sulfonyl)(palmitoyl)amide to afford compound **4**. All synthesized compounds were purified and characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HRMS) with mass error <1 ppm as shown in Figure **S-1**-**6** in the supporting information, which indicated that compounds **1**-**4** are >99 % pure with the exception of compound **3** with ~ 85% purity.

We formulated NEs (NE **1**-**5**) consisting of a highly refined soybean oil, ethanol, and water, emulsified with a non-ionic surfactant and either compound **5**, the prototype NE surfactant, or each of the ZI surfactants (compounds **1**-**4**) following previously optimized protocol.[6] The particle size distributions of the NEs are considered important features of these formulations since they require cellular uptake. We first measured particle diameter of each NE by dynamic light scattering (DLS) in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES, pH~7). The average particle diameters (Z_{ave} d) for all five NEs are shown in Figure **2A**. NE size distributions were unimodal with low polydispersity (PdI< 0.25); however, the average droplet size varied, ranging from 250 to 550 nm for 5 NE formulations.

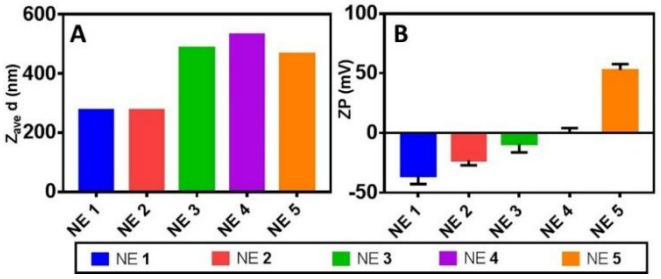


Figure 2. A) Size (Z_{ave} d nm) **B)** ζ-potential of NE formulations **1-5** consist of compounds **1-5**.

The size is within the range of NEs that have been shown to be effective carriers *in vivo*, as reported earlier.[6] NEs **1** and **2** showed a diameter ~ 276 nm. NEs **3**, **4** and **5** had sizes of 486, 531 and 466 nm respectively. Next, we measured the ζ-potential of 5 NE formulations. The NE ζ-potential measurements showed values that ranged from -36 mV to +53 mV as presented in Figure **2B**. As expected, given the charges on each of the ZI, NEs **3** and **4** showed slightly negative (-9.5±6.8 mV) and neutral (0.11±3.9 mV) charges, respectively. NEs **1** and **2** showed negative charges of -36.2±6.6 and -23.1±4.0 mV respectively, whereas **5** had a positive charge of 52.7±5.2 mV. Negative ζ-potential values were also observed for similar ZI (**1** and **2**) micelles by Priebe et al. as sulfobetaine and phosphorylcholine preferentially incorporate anions rather than cations in the interfacial region, resulting in an anionoid micelle.[16]

The induction of a moderate degree of cytotoxicity induced by pathogens or carriers are associated with activation of innate immunity and inflammation.[17] Therefore, we screened the NEs **1**-**5** for cytotoxicity with KB cells. Previous studies reported that cell types had very little impact on the relative cytotoxicity of each NE, and the trends in IC50 values observed in one cell type were similar in all others.[6] Toxicity was evaluated in either 100% cell media or a 1:1 mixture of Opti-MEM and cell media (V:V) over a 50,000-fold range of NE concentrations. From these studies the 50% inhibitory concentration (IC50) for each formulation was identified after 24 hrs of NE exposure, employing a XTT cell viability assay (Figure **3**). NEs **3** and **4** showed no detectable cytotoxicity in any NE concentration tested with either 100% cell media or the 1:1 mixture of Opti-MEM and cell media. NEs **1** and **2** showed no detectable cytotoxicity in 100% cell media at any tested NE concentration. However, both (NEs **1** & **2**) showed an IC50 of ~ 0.21% and 0.17% NE concentration respectively in the

mix of cell media and Opti-MEM. NE **5** containing the cationic surfactant (compound **5**) showed IC50 of 0.16% and 0.08% NE concentration respectively in 100% media and the mixture of Opti-MEM and cell media. Overall these cytotoxicity studies in KB cells showed that NEs (**1**-**4**) that consist of ZI surfactants (compounds **1**-**4**) are either not cytotoxic (NEs **3**-**4**), or less cytotoxic (NEs **1**-**2**) than NE **5** containing the cationic surfactant (compound **5**).

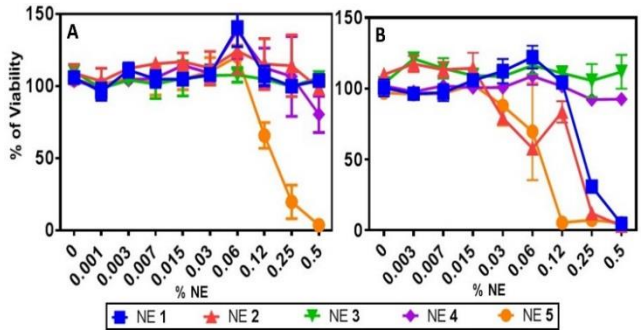


Figure 3. NE formulations induced cytotoxicity for KB cells by NEs **1**-**5** in **A)** 100% cell media, **B)** 1:1 mixture of Opti-MEM and cell media.

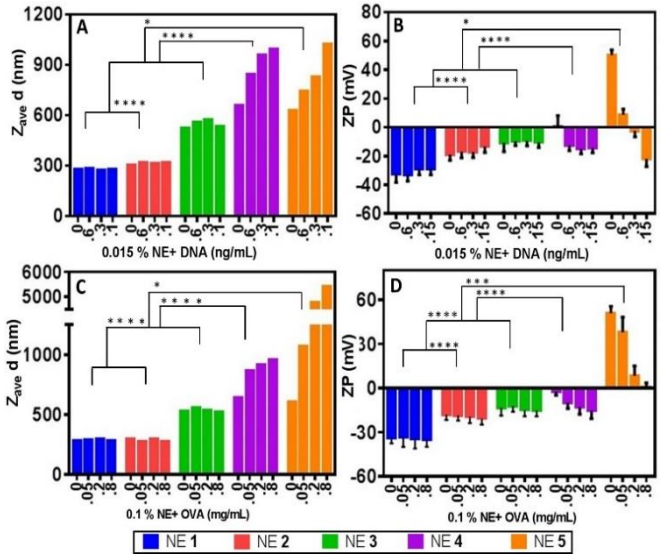


Figure 4. NE and plasmid interaction studies: **A**) size distribution and **B**) ζ-potential of 0.015 % NEs (**1**-**5**) alone (control) or incubated mixture of 0.015 % NEs (**1**-**5**) and 0.6, 0.3 and 0.15 ng/mL plasmid respectively in the measured solution. NE and OVA interaction studies: **C**) size distribution and **D**) ζ-potential of 0.1 % NEs (**1**-**5**) alone (control) or incubated mixture of 0.1 % NEs (**1**-**5**) and 0.8, 0.2 and 0.05 mg/mL OVA respectively in the measured solution. There is a statistically significant difference between group means as determined by one-way ANOVA ($p < 0.05$) with an except between group means of NEs **4** and **5** (not significant). Size distributions were unimodal with low polydispersity (PdI< 0.25).

To determine the interaction between NE and plasmid/antigen (OVA), we measured particle size and surface charge, by DLS and ZP measurement, after the addition of plasmid/OVA at various concentrations to the NEs (**1**-**5**). The mixture (NE+plasmid/OVA) of NEs **1**-**3** didn't show any noticeable change in size and ζ-potential with increasing concentrations of plasmid or OVA. However, Figure **4A** and **4C** showed that Z_{ave} increased for the NE+plasmid/OVA mixture of NEs **4** and **5,** over the same plasmid or OVA concentration range, indicating complex formation with NEs **4** and **5**. Additionally, as the concentration of negatively charged plasmid and OVA increases in the mixture, ζ-potentials for plasmid/OVA and NEs **4**/**5** mixture decreased as shown in Figures **4B** and **4D** which further confirmed the incorporation of plasmid and OVA to the NE formulations **4** and **5**. Interestingly, the magnitude of the particle size increase for the complex of NE **5** with OVA was much greater than that of NE **4** with OVA, as the concentration of OVA was increased, likely due to the formation of aggregates as the NE cationic charge becomes neutralized upon complex formation. There is a statistically significant difference between group means of Z_{ave}/ζ-potentials of mixture (NE+ plasmid/OVA) as determined by one-way ANOVA ($p < 0.05$) and shown by */**/***/**** with an except between group means of NEs **4** and **5** (not significant). Size distributions were unimodal with low polydispersity (PdI< 0.25).

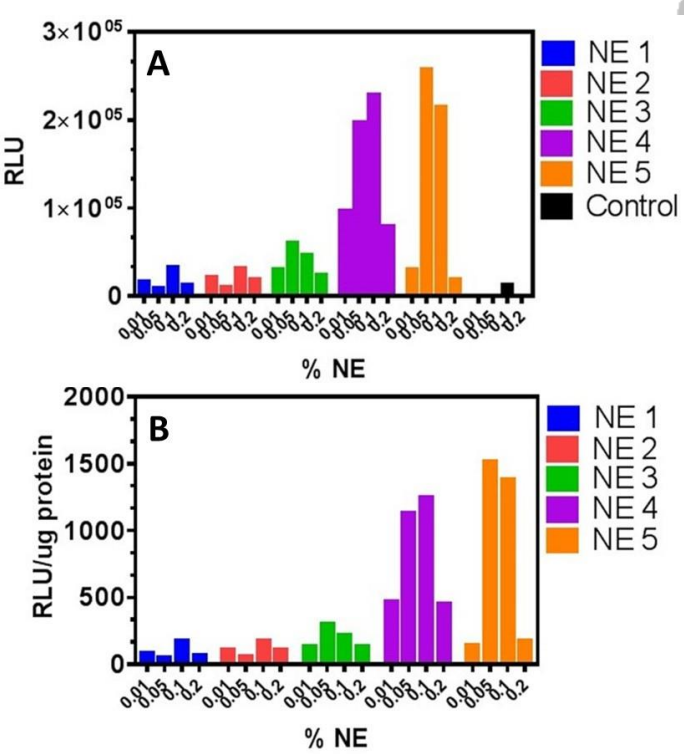


Figure 5. A) NE induced transfection by NEs **1**-**5** at different concentrations in KB cells in a 1:1 mixture of Opti-MEM and cell media (V:V) over a 50,000-fold range of NE concentrations. There is a statistically significant difference between group means as determined by one-way ANOVA ($p < 0.05$) and shown by **/*** except between group means of NE **4** and **5** (not significant). **B**) RLU/µg protein vs % NE induces transfection by NEs **1**-**5** at different concentrations in KB cells in a 1:1 mixture of Opti-MEM and cell media (V:V) over a 50,000-fold range of NE concentrations.

Next, the DNA transfection efficacy of these five formulations (NEs **1**-**5**) was tested on KB cells using a luciferase expression plasmid as the reporter system. Luciferase expression was measured as relative light units (RLU) in KB cells transfected with NEs **1**-**5** in a 1:1 mixture of Opti-MEM and cell media (V:V) at various NE concentrations. These experiments were performed in a 1:1

mixture of Opti-MEM and cell media (V:V) because initial studies showed that a higher transfection efficacy was achieved in this mix than in undiluted cell media which contains serum. These results were analysed by one-way ANOVA which show that transfection efficacies achieved by NEs **4** and **5** at different concentrations are significantly different from that of NEs **1**-**3** ($p < 0.05$). However, there was no statistically significant difference between group means of the transfection efficacies of NEs **4** and **5** at different concentrations as determined by one-way ANOVA. Similarly, the transfection efficacies of NEs **1**-**3** are not statistically significantly different. To further confirm the trend of transfection by NEs **1**-**5** at different concentrations in KB cells we measured the total cell lysate protein concentration (µg/mL, Figure S-7 in the supporting information) and calculated RLU/ µg protein. Figure 5B displays the RLU/ug protein vs % NE and indicates a similar trend to what was seen in our transfection data (Figure 5A).

Results indicate that NEs **4** and **5** achieved the highest transfection efficacies, with NE **5** having the highest transfection efficacy, ~ 30% higher than that of NE **4,** at 0.05% NE concentration. This trend is reversed (6% lower) at 0.1% NE concentration. NEs (**1**-**5**) consist of ionic surfactants (compounds **1**-**5**) and same nonionic surfactants at same ratios (W:W) were produced following previously optimized protocol. Interestingly, because the molecular weight of compound **4** is 1.23 times higher than that of compound **5**, the molar concentration of compound **4** was ~19% less than that used with compound **5** in NEs **4** and **5** formulations respectively. NE **5,** which showed the highest efficacies at 0.05 % and 0.1% NE concentration respectively, fell off at the highest NE concentration, presumably due to cell toxicity. We also noticed that NE **4** showed the lowest transfection efficacy at 0.2% NE concentration. This led us to speculate that besides toxicity, other factors may be involved because no noticeable toxicity of NE **4** was observed at that concentration. In contrast to this, NEs **1**-**3,** consisting only of a ZI head group, showed much lower transfection efficacies than NEs **4** and **5**. There was no difference in transfection with either NEs **1** or **2,** even though NE **2** has a positive outer layer whereas NE **1** has a negative charge in the outer layer; yet both showed negative ζ-potential. While NEs **1** and **2** showed minimal cytotoxicity at higher concentrations as compared to NE **5**, the most interesting result is with NE **4**. This compound is comprised of a ZI head with a positive charge in the outer layer that showed neutral ζ-potential and no cytotoxicity, but its higher cellular uptake resulted in high transfection efficacy. In contrast, NE **3** with a ZI head that has a positive charge in the outer layer with additional hydrophobicity due to the presence of aromatic ring showed slightly negative ζ-potential and no cytotoxicity, but despite this it had minimal cellular uptake and transfection efficacy.

Antigen uptake by epithelial and antigen presenting cells is critical for immunogenicity of carriers through antigen processing and presentation. Since NEs **4** and **5** showed high transfection efficacies in KB cells, we evaluated their ability to facilitate cellular uptake of a protein antigen in mouse lung epithelial cells. The ability of NEs **4** and **5** to enhance antigen uptake was evaluated in the TC-1 cell line. To differentiate intracellular uptake from simple cell adhesion, a self-quenched fluorescently labelled OVA (DQ-OVA) was employed as the antigen. The DQ-OVA fluorescence remains quenched until the antigen undergoes proteolytic processing inside the cell in the endosome. The optimal NE concentration range for uptake enhancement was identified for both NEs **4** and **5** and the intracellular location of antigen was confirmed by confocal fluorescence microscopy (Figure **6**). However, NEs **2** (Figure S-8 in supporting information) and control (Figure 6 I) showed no transfection efficacy in KB cells.

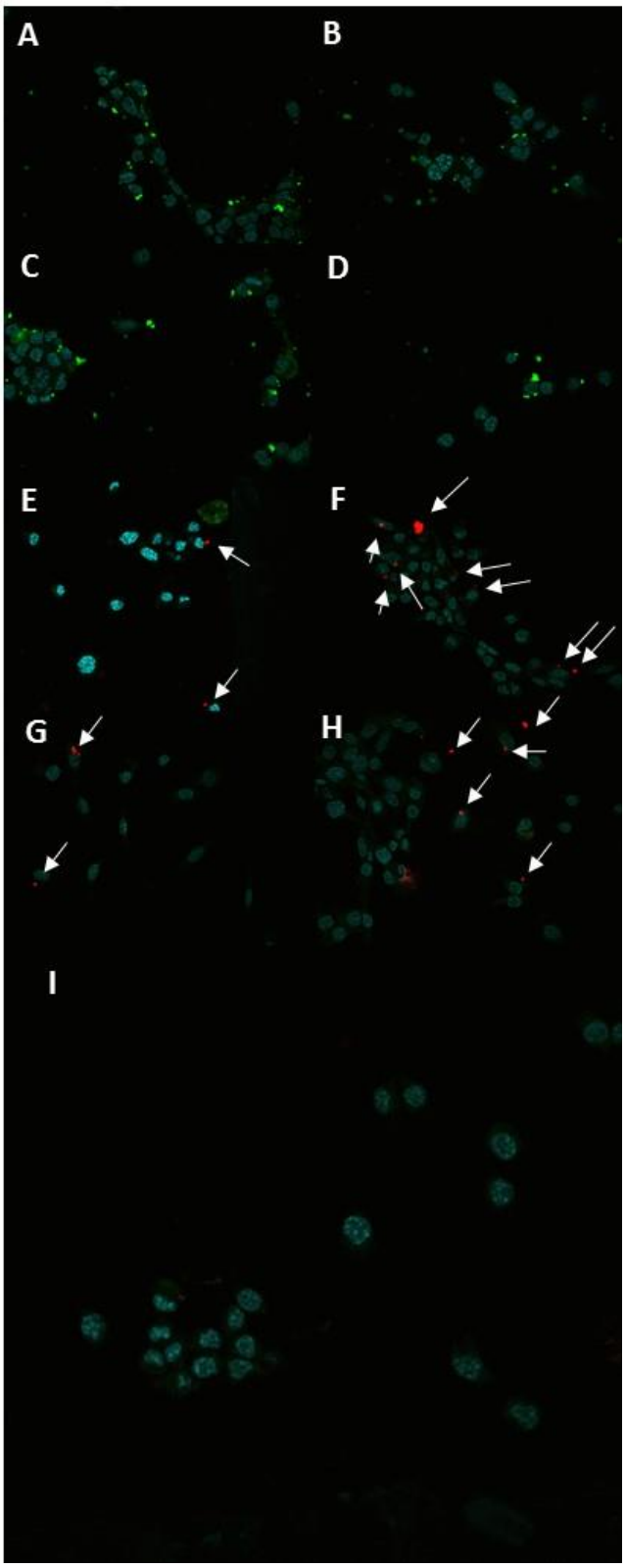


Figure 6. Antigen uptake studies: **A)** NE **4** 0.05% DQOVA. **B)** NE **5** 0.05% DQOVA. **C)** NE **4** 0.01% DQOVA. **D)** DQOVA **E**) NE **4** 0.01%

A647OVA. **F)** NE **4** 0.05% A647OVA. **G)** NE **5** 0.01% A647OVA. **H)** NE **5** 0.05% A647OVA. **I**) A647OVA.

In conclusion, this work demonstrates that NE **4,** consisting of ZI surfactants, can effectively mediate cellular uptake of antigen despite not having cytotoxicity as compared to cationic emulsions (NE **5**). The engineering of this unique molecule (compound **4**), consisting of a hydrophobic tail and ZI head, with a positive charge in the outer layer could be a promising approach to enhance the carrier efficacy while improving tolerability. However, NE **3** that has positive charge in the outer layer with additional hydrophobicity due to the presence of aromatic ring had minimal cellular uptake and transfection efficacy. This study demonstrates the potential implications for developing a nontoxic, next generation, carrier for nanoemulsion-based vaccines.

Supporting information summary

The detail experimental procedures of DLS and transfection studies, synthesis of surfactants, preparation of nanoemulsions are given in the supporting information. Supporting information also provides NMR and mass spectra of surfactants (Figure S1-S6), image corresponds to antigen uptake by NE **2** (Figure S7) and graph corresponds to total cell lysate protein concentration of NE induced transfection by NEs **1**-**5** at different concentrations (Figure S8).

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Keywords: Carrier • Nanoemulsion • Surfactant • Vaccine • Zwitterion

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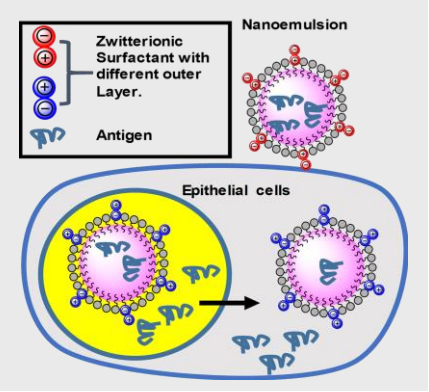
COMMUNICATION

We report a nanoemulsion consists of a zwitterionic surfactant can effectively mediate cellular uptake of antigen despite not having cytotoxicity as compared to a NE consists of a cationic surfactant. ZI surfactant consists of a polar head, with positive charge in the outer layer has implications of developing next generation carrier to enhance the carrier's efficacy while improving tolerability for the development of NE-based vaccine.



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