Chemistry SELECT

Supporting Information

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

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Supporting Information

Experimental Section

General

All the chemicals were purchased from Sigma-Aldrich or Fischer Scientific, unless otherwise specified. The chemicals were used as received. The yields of the compounds reported here refer to the yields of spectroscopically pure compounds after purification.

Synthesis of carriers.

3-(cetyldimethylammonio)-1-propanesulfonate (Compound **1**). An acetone solution (20 mL) of 1,3-propane sultone (3.80 g) was added into a solution of N,N-dimethylhexadecylamine (8.10 g) under continuous stirring. After refluxing for 15 h, solid was collected by filtration and recrystallized from the mixture of methanol-acetone. (Yield 90%) was obtained as a white solid. ^{1}H NMR (400 MHz, CD_3OD), ppm: 4.568 (s 1H), 3.490 (m, 2H), 3.072 (s, 6H), 2.857 (t, 2H), 2.175 (m, 2H), 1.777 (m, 2H), 1.280 (s, 26H), 0.874 (t, 3H). ^{13}C (100 MHZ, CD_3OD): 65.578, 63.805, 51.233, 33.061, 30.775, 30.748, 30.722, 30.641, 30.5777, 30.463, 30.225, 27.422. 23.724, 23.496, 19.899, 14.444. MS: m/z calculated for $C_{21}H_{46}NO_3S$ (M): $[M + H]^+$ 392.3193; found: 392.3192

Hexadecyl (2-(trimethylammonio)ethyl) phosphate (Compound **2**). Hexadecanol (60.61 g, 0.25 mol) and triethylamine (38.33 mL, 0.275 mol) are dissolved in anhydrous 300 mL THF. The solution is added dropwise with vigorous stirring to a solution of phosphorous oxychloride (27.96 mL, 0.3 mol) in 100 mL THF precooled by an ice-salt bath at -10 °C. After the addition of hexadecanol, the reaction mixture is kept at room temperature with stirring for another 4 hours. The solution was used directly for the next step. A solution of ethanolamine (18.11 mL, 0.3 mol) and triethylamine (83.63 mL, 0.6 mol) in 200 mL THF is added dropwise under vigorous stirring to the reaction mixture of step one at -10 °C. After the addition of ethanolamine, the reaction mixture is kept at room temperature with stirring for another 4 hours. Then the reaction mixture was filtered by suction to remove precipitated triethylamine hydrochloride. The clear and slightly yellow filtrate is used directly for the next step. The filtrate of step two is mixed with 100 g acetic acid and 150 mL distilled water. Then the mixture is heated at 70 °C under stirring for 4 h. A lot of insoluble white solid precipitated from the solution. Then the suspension was condensed by azeotropic distillation with ethanol, and the product is precipitated by acetone. Then the solid is collected by suction filtration and washed by acetone for two more times. The obtained hexadecylphosphoethanolamine is dissolved in 200 mL methanol and 200 mL dichloromethane. Methyl iodide (155.6 mL, 2.5 mol) and 100 mL of methanol containing NaOH (30 g, 0.75 mol) were added to the solution. The mixture was stirred in dark at 35 °C for 72 hours. The insoluble substance was removed from the solution by suction filtration and a transparent yellowish solution was obtained. The solution was passed through an Amerlite MB 20 mixed-bed ion resin column and precipitated from acetone. The white solid was collected by suction filtration and dried completely under vacuum.

TLC showed a single spot (yield 57.9 %. ^{1}H-NMR (400 MHz, CD_3OD) 4.238 (m, 2H), 3.860 (q, 2H), 3.614 (m, 2H), 3.210 (s, 9H), 1.625 (p, 2H), 1.387 (t, 2H), 1.279 (bs, 24H), 0.891 (q, 3H). ^{13}C (100 MHZ, CD_3OD): 67.533, 67.505, 67.465, 67.430, 67.401, 66.935, 66.876, 60.267, 60.219, 54.717, 54.678, 54.640, 33.065, 31.928, 31.855, 30.787, 30.752, 30.492, 30.466, 26.939, 23.727, 14.497. MS: m/z calculated for $C_{21}H_{47}NO_4P$ (M): $[M+H]^+$ 408.3237; found: 408.3238.

4-(hexadecyloxy)benzenesulfonamide. To a solution of 4-hydroxybenzenesulfonamide (1.5 g, 8.66 mmol, 1.4 equiv.) in the mixture of acetone and DMF (3:1, V: V), K_2CO_3 (1.2 g, 8.66 mmol, 1.4 equiv.) was added and allowed to stir for 1 h. Then 1-iodohexadecane (2.18 g, 6.2 mmol, 1 equiv.) was added and the solution mixture was stirred for 24 h at 60 °C. The mixture was concentrated; the residue was purified by flash chromatography over silica gel with n-hexane–EtOAc (4:1) to give the desired compound as white solid (yield 71%). ^{1}H-NMR (400 MHz, $CDCl_3$) 7.825 (d, 2H), 6.940 (d, 2H), 4.712 (s, 2H), 3.986 (t, 2H), 1.779 (p, 2H), 1.550 (s, 2H), 1.431 (p, 2H), 1.236 (bs, 22H), 0.857 (t, 3H). ^{13}C (100 MHZ, $CDCl_3$): 162.625, 133.241, 128.562, 114.667, 68.502, 31.908, 29.674, 29.639, 29.570, 29.532, 29.343, 29.324, 29.000, 25.927, 22.675, 14.104. MS: m/z calculated for $C_{22}H_{39}NO_3S$ (M): $[M + H]^+$, $[M + NH4]^+$: 398.2724, 415.2989; found: 398.2729, 415.2986.

((4-(hexadecyloxy)phenyl)sulfonyl)(4 (trimethylammonio)butanoyl)amide (Compound **3**). (3-carboxypropyl)- trimethylammonium chloride (400 mg, 2.2 mmol, 1 equv.) was subjected to react with thionyl chloride (1 mL) at RT for 1h. Then excess thionyl chloride was removed under reduced pressure and subsequently, added 4-(pentadecyloxy)benzenesulfonamide (877.5 mg, 2.2 mmol, 1 equv.) and potassium iodide (220 mg, 1.32 mmol, 0.6 equv.) in acetonitrile (1 mL). The mixture was refluxed for 36h. The residue was purified by flash chromatography over silica gel with $CHCl_3$: MeOH: water (80:18:2) to give the desired compound as yellowish white solid (yield 70%) with > 85% purity. ^{1}H-NMR (500 MHz, CD_3OD) 7.910 (d, 2H), 7.040 (d, 2H), 4.053 (t, 2H), 3.675 (s, 2H), 3.092 (s, 9H), 2.890 (s, 1H), 2.649 (s, 1H), 2.364 (t, 2H), 1.974 (m, 2H), 1.788 (m, 2H), 1.474 (m, 2H), 1.280 (bs, 22H), 0.889 (t, 3H). ^{13}C (100 MHZ, $CDCl_3$): 172.106, 164.828, 132.073, 131.437, 115.486, 69.698, 66.610, 55.109, 53.523, 33.056, 30.756, 30.739, 30.684, 30.455, 27.066, 23.720, 18.700, 14.434. MS: m/z calculated for $C_{29}H_{52}N_2O_4S$ (M): $[M + H]^+$:525.3721; found: 525.3725.

((3-chloropropyl)sulfonyl)(palmitoyl)amide. To a mixture of Palmitic acid (1.06 g, 4.12 mmol, 1 equiv.), EDC·HCl (948.4 mg, 4.95 mmol, 1.2 equiv.), DMAP (504 mg, 4.12 mmol, 1 equiv.) in DCM in as ice bath, a solution of trimethylamine (500 mg, 4.95 mmol, 1.2 equiv.) and 3-chloropropane-1-sulfonamide (649.8 g, 4.12 mmol, 1 equiv.) in DCM was added slowly. The mixture was stirred for 6h and then washed with water and organic layer was collected, dried and purified by flash chromatography over silica gel with n-hexane–EtOAc (1:1) to give the desired compound as white solid (yield 71%). ^{1}H-NMR (400 MHz, $CDCl_3$) 3.637 (m, 4H), 2.310 (m, 4H), 1.639 (p, 2H), 1.549 (bs, 4H), 1.234 (bs, 20H), 0.859 (t, 3H). ^{13}C (100 MHZ, $CDCl_3$): 168.236, 47.567, 38.757, 33.233, 28.504, 26.264, 26.241,

26.208, 26.147, 25.963, 25.940, 25.790, 25.531, 22.943, 21.054, 19.273, 10.702. MS: m/z calculated for $C_{19}H_{37}ClNO_3S$ (M): 394.2188; found: 394.2181.

palmitoyl((3-(trimethylammonio)propyl)sulfonyl)amide (Compound **4**). The ((3-chloropropyl)sulfonyl)(palmitoyl)amide (700 mg, 1.78 mmol) was dissolved in trimethylamine-EtOH (33 wt%, 1.16 mL). After being stirred at 50 °C for 36h the residue was purified by flash chromatography over silica gel with chloroform/methanol/water (80:18:2, V: V) to afford white solid (65% yield). ^{1}H-NMR (500 MHz, CD_3OD): 3.518 (m, 2H), 3.339 (t, 2H), 3.145 (s, 9H), 2.221 (m, 4H), 1.599 (m, 2H), 1.279 (bs, 20H), 0.891 (t, 3H). ^{13}C (125 MHZ, CD_3OD): 182.255, 66.403, 66.387, 66.371, 53.806, 53.783, 53.760, 49.885, 40.019, 32.965, 30.675, 30.638, 30.593, 30.507, 30.491, 30.331, 27.124, 23.603, 19.353, 14.290. MS: m/z calculated for $C_{22}H_{47}N_2O_3S$ (M): 419.3302; found: 419.3308.

Cell Lines. Cell lines were purchased from American Type Culture Collection, Manassas, VA and demonstrated to be free of mycoplasma contamination by RT-PCR (PCR Mycoplasma Detection Set, Takara Bio Inc.). Cell lines used included TC-1(epithelial), and KB. TC-1 cells were grown in RPMI 1640 media with L-glutamine (Corning), containing 10% heat-inactivated FBS (HI-FBS) (Gemini), 1 x nonessential amino acids, 10 mM HEPES, 100 IU penicillin, and 100 μg/mL streptomycin (Gibco). KB Cells were grown in RPMI 1640 media with L-glutamine and phenol red, without folic acid and HEPES, containing 10% FBS & 1% Pen/Strep.

Preparation of Nanoemulsions. Nanoemulsions were prepared by high-speed emulsification of ionic and nonionic surfactants, ethanol (200 proof), soybean oil and purified water using a high-speed homogenizer. A series of NEs consist of ionic surfactants (compound **1**-**5**) and same nonionic surfactants at same ratios (W: W) were produced following previously developed protocol. No changes in physical appearance of NEs was observed over more than a year at room temperature. Surfactant blend ratios of the NEs are annotated according to the ratio of ionic to nonionic surfactants. For example, a CPC/Tween80 NE annotated as 1:6 contains one-part CPC to 6 parts Tween 80 (by weight). At the end of homogenizing the mixture at 10,000 RPM for 30 mins ice bath to keep the temperature $\leq$ 10 ^{o}C, a milk like liquid was obtained (100 % solution) which was further diluted into 60 % solution (W: W) using water. Those NE formulations were stored at 4 ^{o}C.

Dynamic Light Scattering (DLS) and Zeta Potential (ZP). DLS and ZP measurements were performed successively for the same sample on a Zetasizer Nano-ZS (Malvern Instruments Ltd.). 0.1%/ 0.15% NE (w/v) was mixed in 1 mM HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]- ethanesulfonic acid), pH 7, at RT for 2 min before measurement. Particle are expressed as average diameter (Zave d nm). The reported error for size measurements was calculated as standard deviation from measured polydispersity index (PdI) values. The error for ZP measurements is reported as actual measurement zeta deviation values.

Cellular Toxicity Screening. Cellular toxicity was evaluated using XTT Cell Proliferation Assay. We first seeded ~200 μL KB cells (50, 000 cells/well) from a stock 2.5 x 10^5 cells

in 40 mL of media on 96-well plates overnight at 37 °C overnight. Next morning media was removed from the wells and 90 μL of Opti-MEM or cell media was added to each well. Separately, serial dilutions of NE were prepared in PBS, spanning a 50,000-fold concentration range (0.5% – 0% NE (w/v)). Subsequently, 20 μL of the NEs (1-**5**) of different dilution were added to the respective wells, mixed well and incubated for 4h at 37 °C. Then, 90 μL of cell media was added to each well, mixed well and incubated for 24h more h at 37 °C. Each condition was run in duplicate. Supernatant was aspirated, and cells were washed with 150 μL PBS. To perform XTT, added 50 μL of warm PBS to each well then added 30 μL of XTT working reagent, mixed on plate mixer and incubated in TC incubator for 6 h before reading. Luminescence was measured at 492 nm and at 690 nm on a PHERAstar plate reader (BMG LabTech). The IC50 was defined as the NE concentration (% w/v) at which there is 50% cell viability after 24 h of treatment.

DNA Transfection. First, 2.5 x 10^5 KB cells were seeded in FA free media in 24 well plates in 500 μL/well and incubate for 37 ºC for 24 h. After 24h, media was removed, and each well was rinsed with 500 μL of PBS, followed by addition of 200 μL of KB Opti-MEM. Separately, we prepared NE and pLuc DNA at a 10X conc. of the lowest conc. used for the experiment in Opti-MEM reduced serum medium. Then we prepared NE/pLuc DNA mixture, at 5x concentration, by mixing equal volumes of the NE and pLuc (30 μL of 10X conc.) or nothing 10X mix (controlled) and incubated for 15 min at RT. The cells were transfected with 50 μL above solution (mixture of NE and pLuc DNA). After 4 h of incubation with the mixture of NE and pLuc DNA at 37 °C, 250 μL of cell culture media was added and incubated for additional 24 h. At 24 h post transfection, the cells were rinsed and lysed in 100 μL of reporter lysis buffer. At the end the luciferase expression was detected using the luciferase assay system according to the manufacturer's protocol.

Antigen uptake by Confocal Microscopy. TC-1 cells were seeded on 8-well chambered cover glass slides (Lab-Tek) overnight at 37°C. Media was replaced with fresh media containing 0.05% NE with 10 μg/mL DQ-OVA. Cells were incubated at 37 °C for 2 h, and then washed 2x with PBS, fixed in 4% paraformaldehyde, allowed to dry before addition of ProLong Gold with DAPI (Thermofisher scientific). Cells were imaged on an LSM 510-META laser scanning confocal fluorescence microscope (Zeiss) (green fluorescence ex = 488 nm em = 500–530 nm was measured for DQ-OVA).

We used same amount of antigen in all the formulations. The antigen we used, ovalbumin, has been shown to be soluble in essentially all the emulsion-based vaccines we have developed in this concentration, and shown to be adequate to produce an optimal antibody response. Once mixed into the emulsion it is impossible to separate the antigen due to the nature of the formulation. Therefore, by adding similar concentrations of antigen to the emulsion we are using a relevant amount of antigen for the testing of these formulations

Dynamic Light Scattering (DLS) and Zeta Potential (ZP) of plasmid and OVA binding. To prepare sample for NE-DMA studies, we prepared 2%, 1% and 0.5% of NE in PBS from 60% NE. Subsequently, we prepared NE/pLuc DNA mixture by mixing 60 μL

of the respective NE solutions and pLuc DNA (20 ng/mL). Control consist of 1% NE with no pLuc DNA. To measure zeta and size, we prepared 4 solutions in HEPES (pH ~7.0) that consist of **1**). 0.15% NE + 0.6 ng/mL pLuc DNA, **2**). 0.15% NE + 0.3 ng/mL, **3**). 0.15% NE + 0.15 ng/mL and **4**). 0.15% NE + 0 ng/ml (control). Each NE solution and pLuc DNA were added and waited for 10 mins before running Zeta.

For NE-OVA binding study we prepared 20% of NE from 60% NE stock in PBS. Subsequently, we prepared NE/OVA mixture by mixing 40 μL of the NE and various amount of 20 mg/mL of OVA solution in PBS. Control consist of 1% NE with no OVA. To measure zeta and size, we prepared 4 solutions in HEPES (pH ~7.0) that consist of **1**). 0.1% NE + 0.8 mg/mL OVA, **2**). 0.1% NE + 0.2 mg/mL OVA, **3**). 0.1% NE + 0.05 mg/m OVA, and **4**). 0.1% NE + 0 mg/ml OVA (control). Each NE solution and OVA were added and waited for 10 mins before running Zeta.

Statistical Analysis. Statistical analysis was performed with the aid of GraphPad Prism version 7.00 (GraphPad). The one-way ANOVA multiple comparisons tests were employed to evaluate the statistical significance of differences between groups. We obtained P = ~0.001 which is < 0.5. Means with lines between bars and marked with *** (p = 0.001) /** (p = 0.0017) are significantly different.

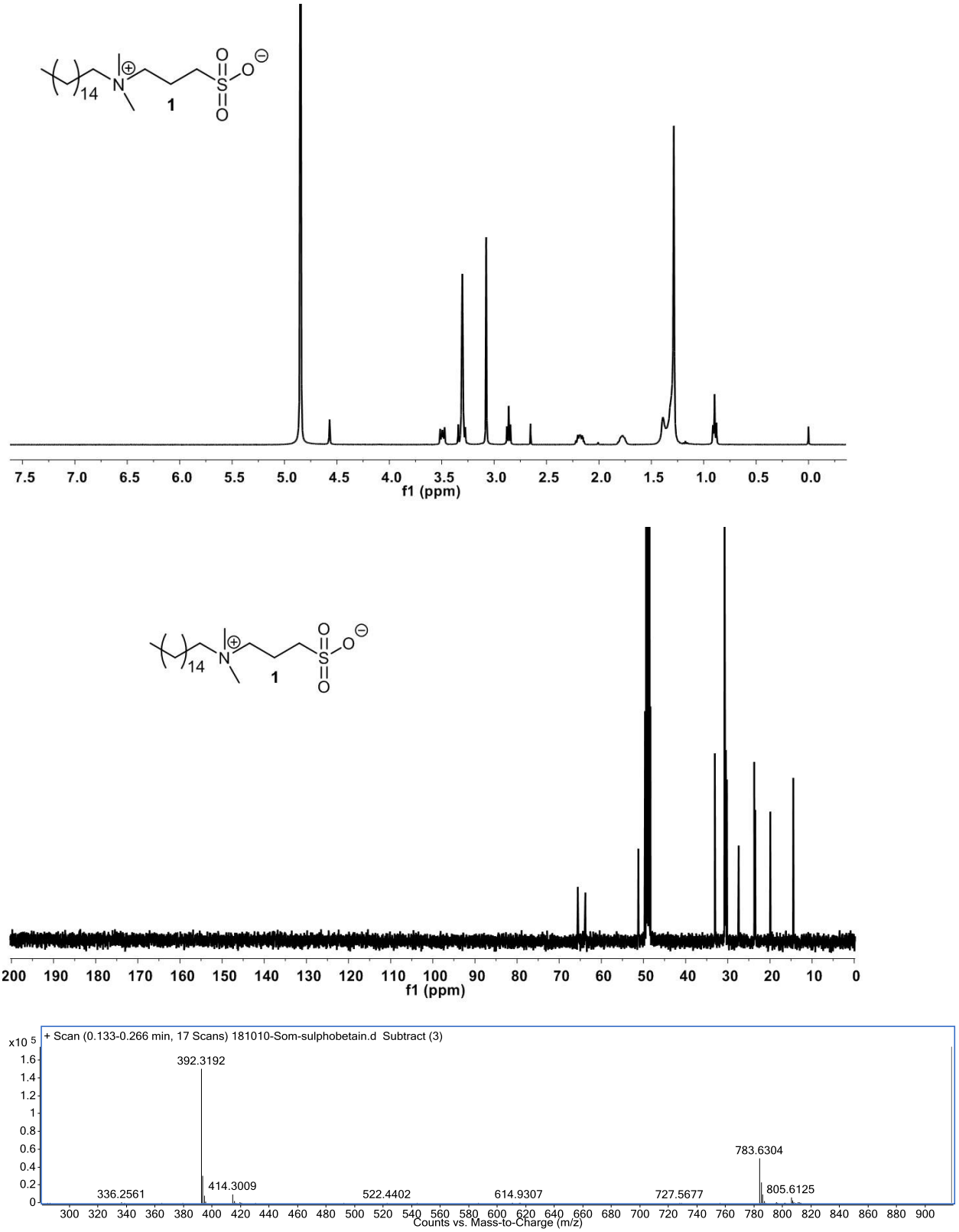
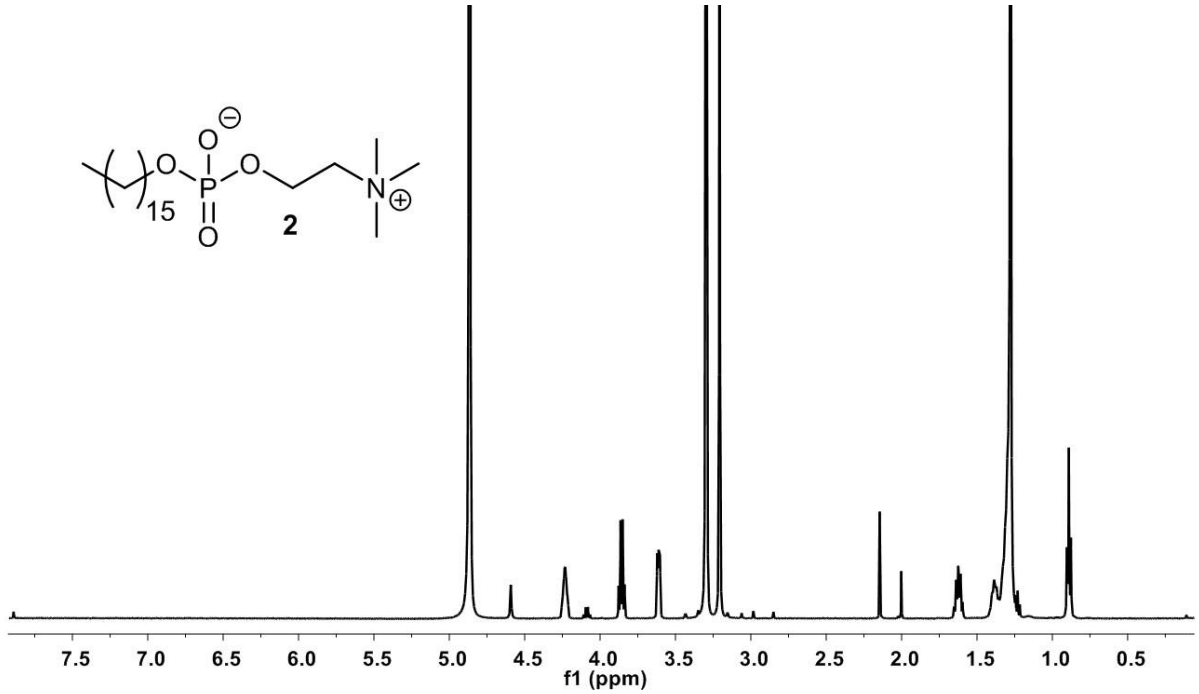
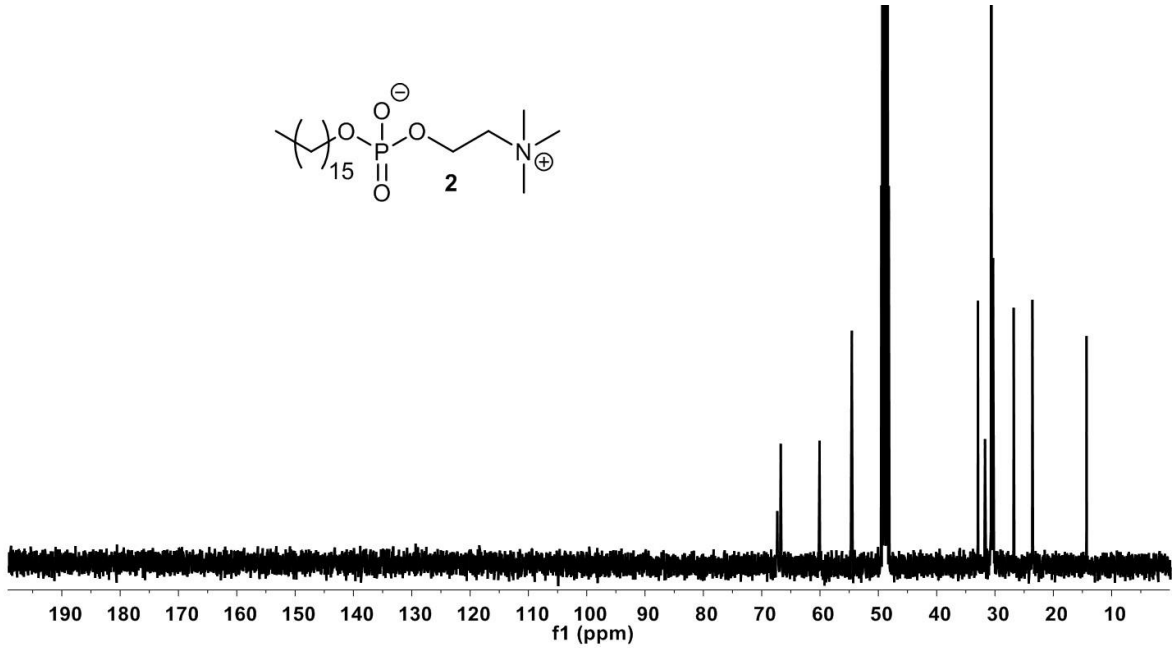


Figure S1: ^{1}H and ^{13}C NMR in CD_3OD and HRMS of compound **1**.





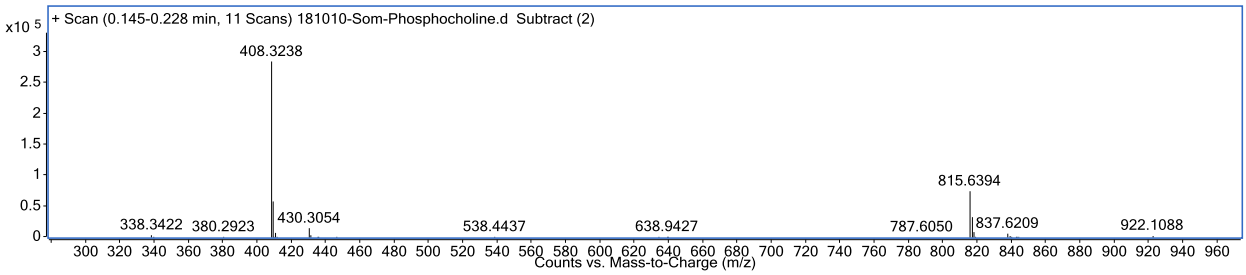
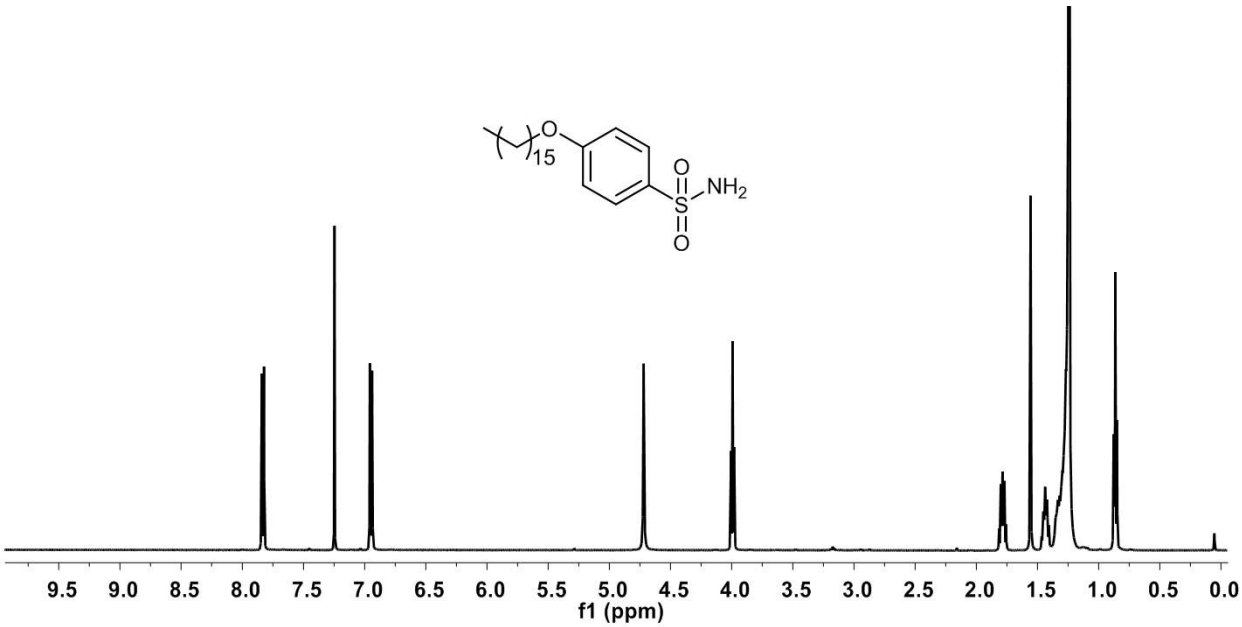
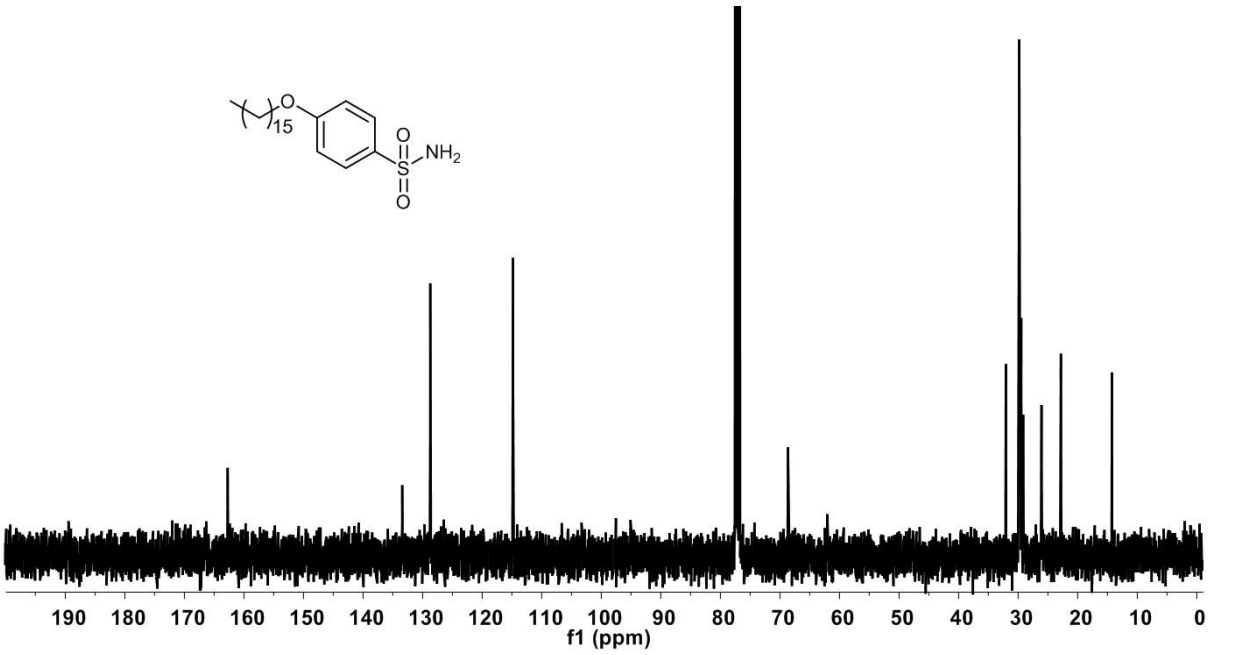


Figure S2: ^{1}H and ^{13}C NMR in CD_3OD and HRMS of compound **2**.





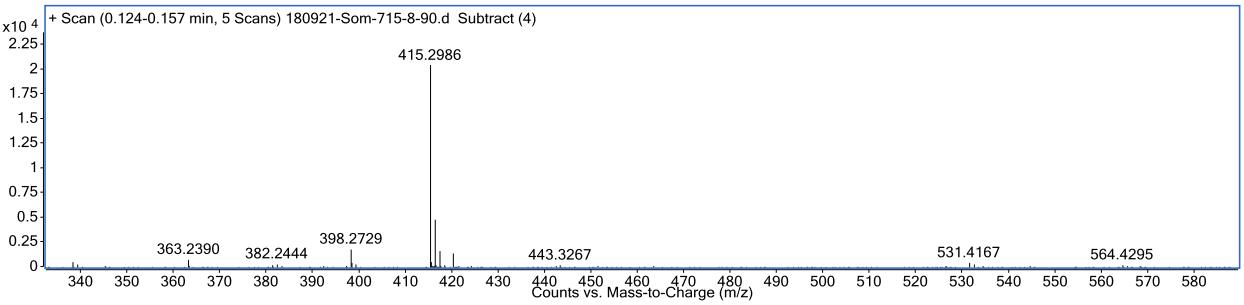
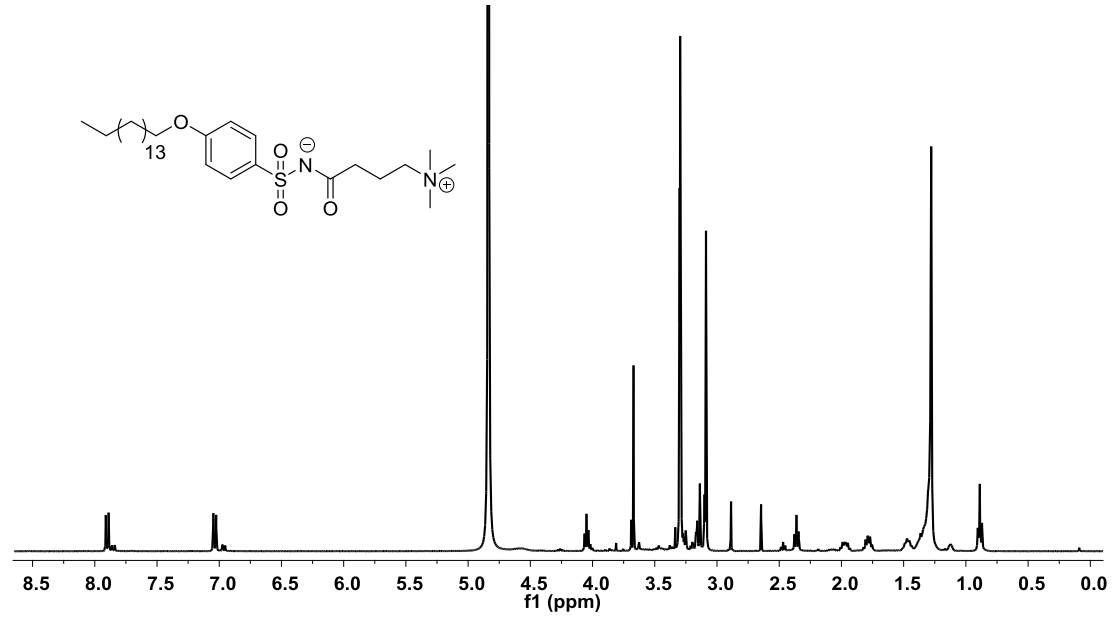
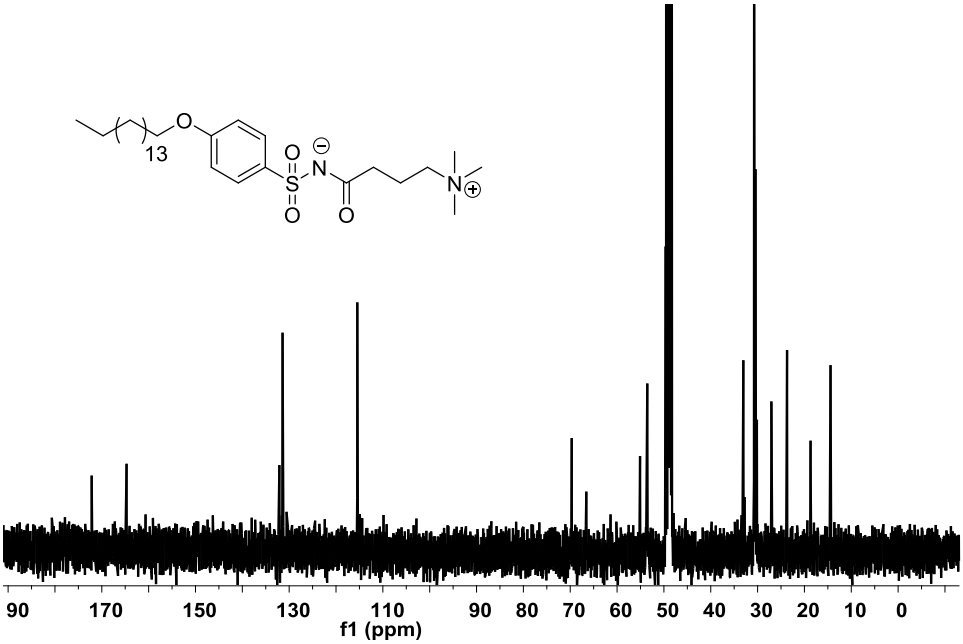


Figure S3: ^{1}H and ^{13}C NMR in $CDCl_3$ and HRMS of 4-(hexadecyloxy)benzenesulfonamide.





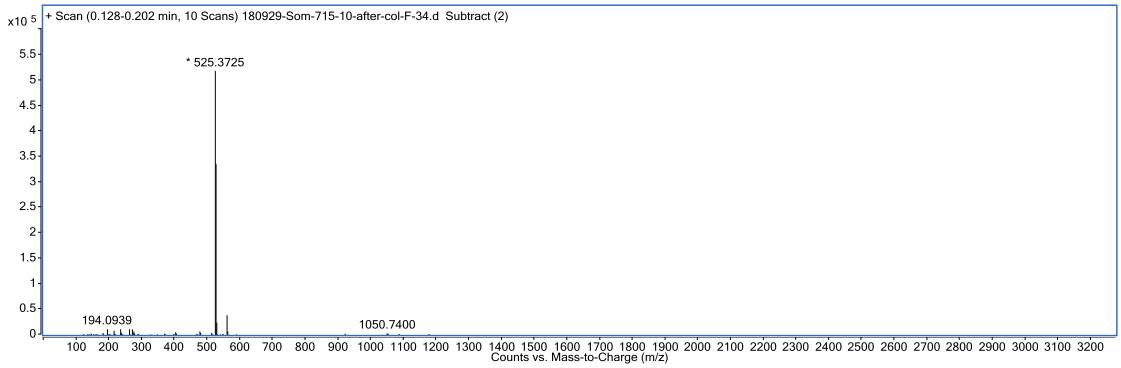
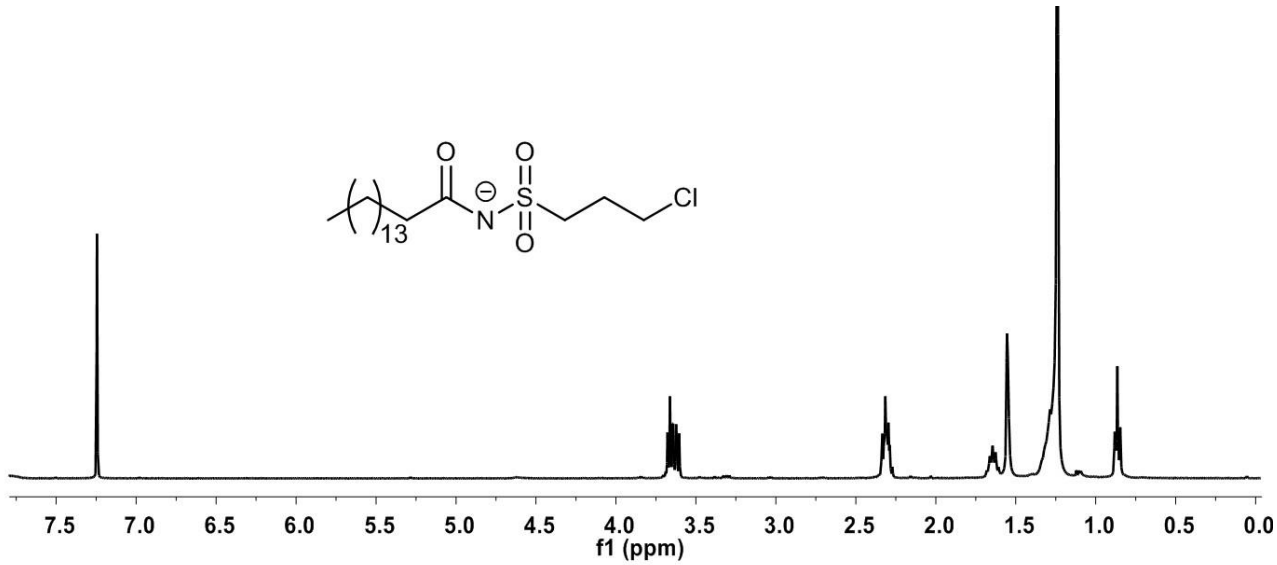
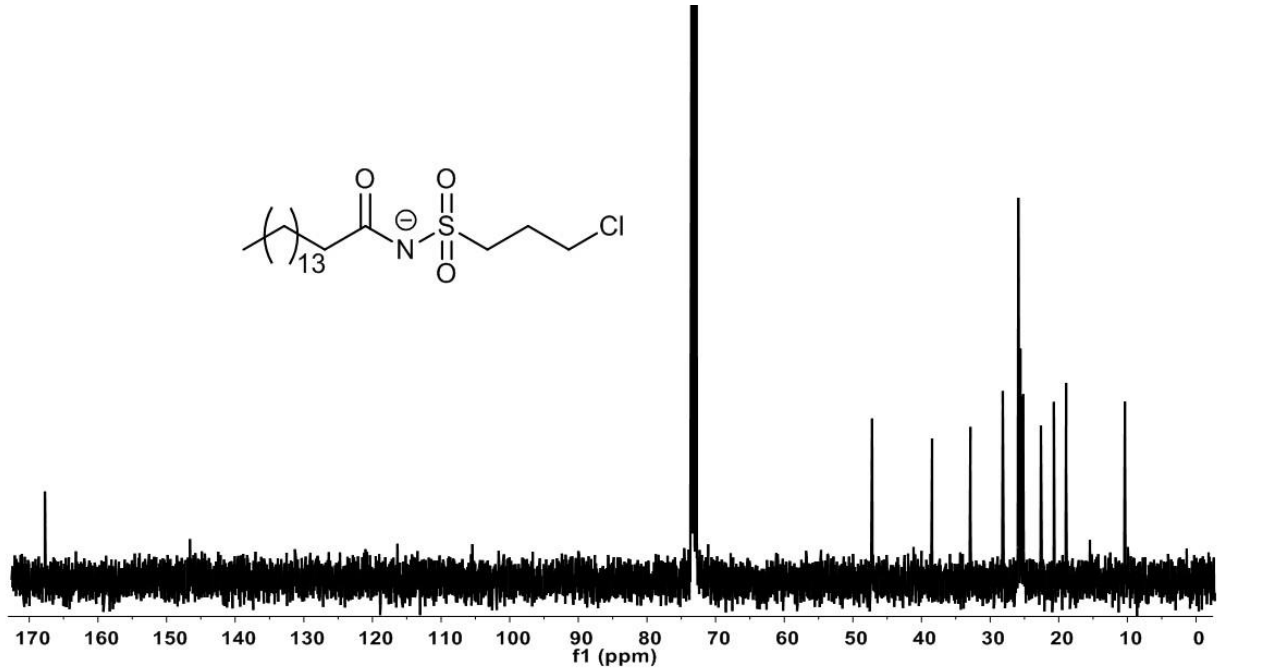


Figure S4: ^{1}H and ^{13}C NMR in CD_3OD and HRMS of compound **3.**





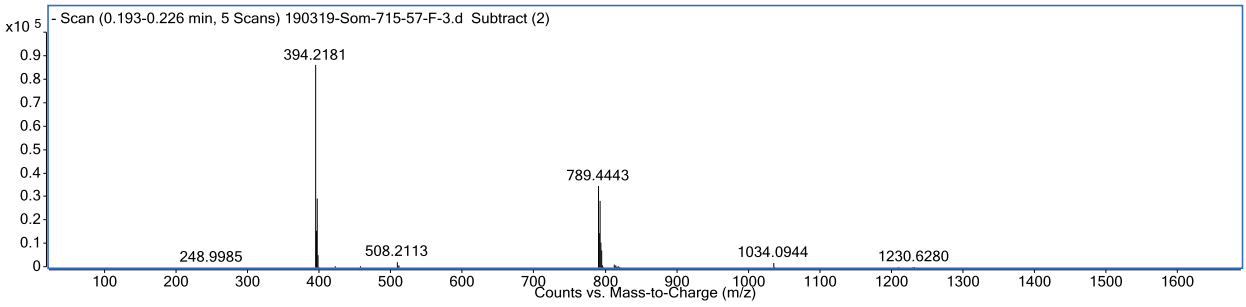


Figure S5: ^{1}H and ^{13}C NMR in $CDCl_3$ and HRMS of ((3-chloropropyl)sulfonyl)(palmitoyl)amide.

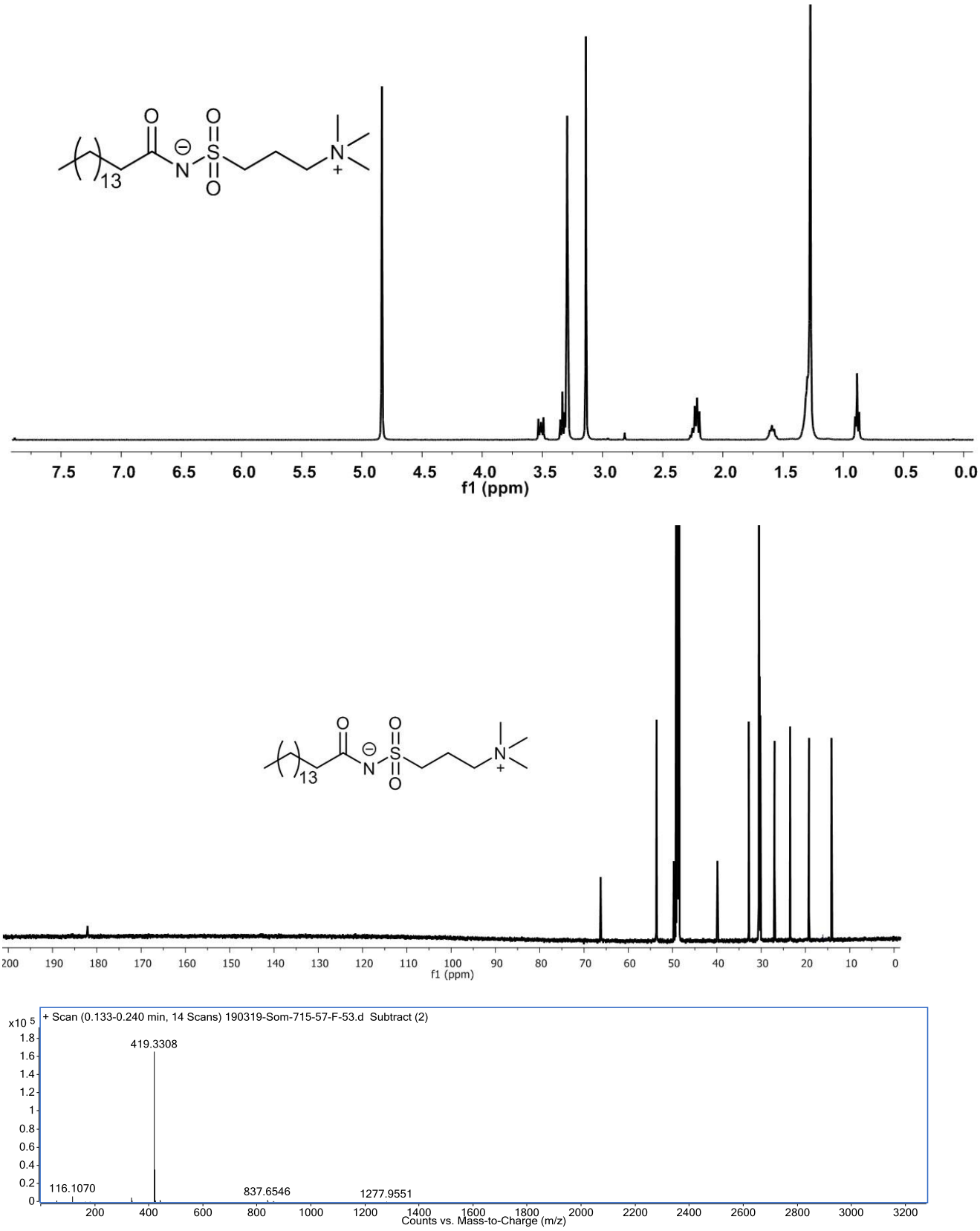


Figure S6: ^{1}H and ^{13}C NMR in CD_3OD and HRMS of compound **4**.

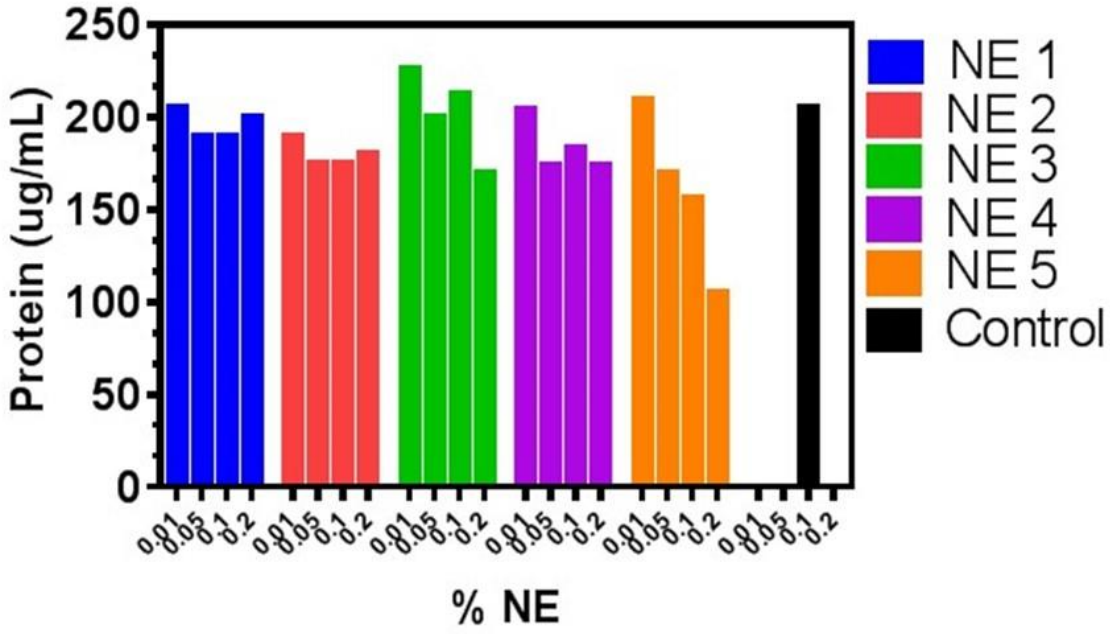


Figure S7: Total cell lysate protein concentration (µg/mL) of NE induced transfection by NEs **1**-**5** at different concentrations in KB cells in a 1:1 mixture of Opti-MEM and cell media (V: V) over a 50,000-fold range of NE concentrations.

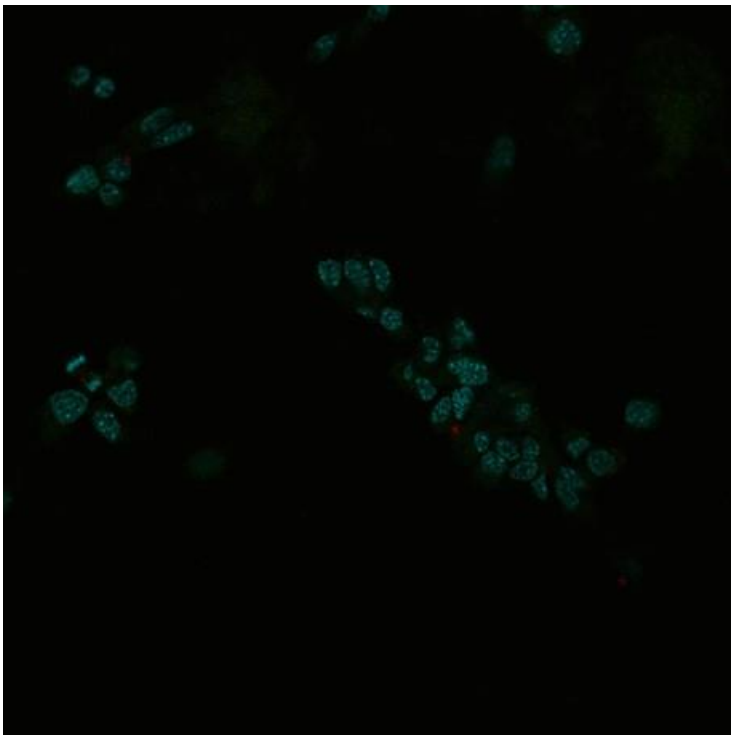


Figure S8: Antigen uptake study of NE **2** 0.01% A647OVA.