Materials and Methods

Animals

B6;129P2-*Omp*^{tm3Mom}/MomJ heterozygous (OMP-GFP) mice were obtained from Jackson Laboratory (Stock #006667) following cryorecovery and were then bred and maintained in an AAALAC-accredited, PHS-assured, environmentally controlled, specific pathogen-free, barrier animal facility. In these mice, green fluorescence protein (GFP) replaces the coding region and a portion of the untranslated region of one copy of the olfactory marker protein gene (OMP). These mice display intense green fluorescence in olfactory neurons from the nose to their axonal terminus in glomeruli of the olfactory bulb. Wild-type controls were from this colony. A Qiagen Dneasy blood and tissue kit was used for DNA isolation, and gel electrophoresis was used to identify genotypes by polymerase chain reaction (PCR) using procedures from the mouse supplier. To reduce the use of animals in this study and because preliminary data were from male mice, all mice used for experimental purposes in this study were male. All procedures were reviewed and approved by the CDC-Morgantown Animal Care and Use Committee.

Chemicals

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was acquired from Sigma-Aldrich (St Louis, MO, product #P2663). 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was acquired from Sigma-Aldrich (St Louis, MO, product #P0763). Lactated Ringer's solution (LRS) was acquired from Patterson Veterinary (Greeley, CO, catalog #078696319). Dulbecco's phosphate-buffered saline (PBS) without calcium chloride or magnesium chloride was from Sigma-Aldrich (St Louis, MO, catalog #D8537). TrophAmine 10% amino acid solution was acquired from Medline Industries (Northfield, IL, catalog #BMGS9341H). Ethanol was acquired from Sigma-Aldrich (St Louis, MO, catalog #E7023-500ml). Mouse albumin was acquired from Innovative Research (Novi, MI, catalog #IRMSA) and tested free of common murine pathogens (IDEXX, IMPACT 1 PCR Panel). Hexagonal boron nitride (HBN) was acquired from Sky Spring Nanomaterials, Inc (Houston, TX). Tetramethylrhodamine anionic lysine-fixable dextran (rhodamine dextran, 3000 MW, catalog #D3308) and Alexa Fluor 680 anionic dextran (3000 MW, catalog #D34681) were acquired from ThermoFisher (Waltham, MA).

Composition and Preparation of SNOT

The SNOT is composed of 1 mg/mL DPPC (as 10 μL of a 1:10 dilution of DPPC in 200 proof ethanol), 0.25 mg/mL DMPC (as 25 μL of a 1:100 dilution of DPPC in 200 proof ethanol), 959 μL/mL LRS and 6 μL/mL TrophAmine. Each was added in that order to a 2 mL tube; the addition of TrophAmine directly to DPPC caused irreversible gel formation. Chemicals were kept at 37°C and the solution was vortexed as each ingredient was added. Finally, the solution was vortexed for 1 minute to thoroughly dissolve all components when additions were completed. The solution was made fresh for each experiment.

Calculation of SNOT Density, Viscosity, and Refractive Index

SNOT was pre-equilibrated to 37°C, dispensed into a tared 10 mL volumetric flask and placed into an analytical balance forgravimetric density determination. For viscosity measurement, SNOT temperature was equilibrated to approximately 25°C before loading into a size 25 Cannon-Fenske Routine viscometer (Cannon Instruments: State College, PA) and then submerged in a 37°C bath for a minimum of 10 minutes before measurement. The efflux time was measured in quadruplicate in seconds and converted to the dynamic viscosity using efflux time, SNOT density, and the viscometer-specific constant (0.0020625 centiStokes/second). The SNOT refractive index was measured via an ISO-9001 certified hand-held refractometer according to the manufacturer's instructions (Reichert Analytical Instruments; Dewpew, NW; Catalog #AR200).

Sonication of Nanomaterials in SNOT

Because of the variations between sonicators, the delivered acoustic power (P) of the sonication equipment (Sonics VibraCell, Model VC505 with 2.75 in cup horn, Newton, CT) was calculated to be 3.34Js^{-1} using published methods.

$$(P) \times (t) / (V) = DSE,$$

where P is the acoustic power (J/s), t is time (seconds), V is volume (mL), and DSE is delivered sonication energy (J/mL). Unless specified, 5 mL solutions were sonicated for 1 minute to achieve 40 J/mL delivered sonication energy (DSE) followed by 1 minute of rest. This was repeated 5 times for a total of 200 J/mL DSE.

Dynamic Light Scattering

Dynamic light scattering (DLS) measurements (Zetasizer Nano ZS, Model Zen3600, Malvern) were used to determine particle hydrodynamic diameter (dH) throughout the study. Sample physical properties for DLS measurements, that is, density, refractive index, and viscosity, were calculated for SNOT. Refractive index and absorption values for HBN were obtained from literature (Table 1). For DLS measurements, a sample volume of 0.5 mL was placed in low volume cuvettes (Zetasizer, ZEN0040; Malvern, United Kingdom). Number distributions were collected and averaged with five replicates. After sonication, dispersed nanomaterials were placed in a water bath at 37°C.

Intranasal Exposures in Mice

Mice were anesthetized using isoflurane anesthesia with induction in a bell jar and maintenance of anesthesia with a SomnoSuite low-flow anesthesia system (Kent Scientific, Torrington, CT). SNOT or dyes dispersed in SNOT were used for intranasal instillation with modification of a previously described procedure. Briefly, the solution was vortexed for approximately 1 minute and 20 microliters were administered intranasally. For intranasal administration, the mouse was placed in sternal recumbency and when surgical plane anesthesia was achieved, the mouse was rotated away from the anesthesia nose cone and a drop of SNOT solution at the end of a sterile micropipette tip was placed in front of one nostril so that normal respiration drew the drop into the nose of the mouse; a second drop was then placed at the end of the micropipette tip in front of the other nostril. When the second drop was inspired, the mouse was rotated back to the nose cone to breathe anesthetic for

approximately 30 seconds and then the procedure was repeated until the entire 20 μL was inspired. Anesthesia continued for approximately 45 seconds following the final inspiration. To prevent aspiration of bedding, mice recovered from anesthesia in a recovery cage lined with absorbent laboratory paper and were transferred back to their home cage when ambulatory.

Pharyngeal Aspiration Exposures in Mice

Lungs of two mice were exposed to SNOT using a previously described pharyngeal aspiration procedure.

Intranasal Exposures to Dextran Dyes

While not typically considered nanomaterials, dextrans are polysaccharides of low toxicity, hydrophilic, and available as conjugates of several dyes. These conjugates are typically between 3000 and 70,000 Daltons, which corresponds to an approximate size of 1 to 20 nm. Some dextran dyes can be up to 2,000,000 Daltons in mass. A 3000 Dalton dextran has a diameter of approximately 2.48 nm. We used 3000 Dalton dextran dyes to track the potential for a nanoscale particle to enter the olfactory bulb after intranasal administration in SNOT because they previously have been shown to undergo neuronal transport in many different conditions, including after intranasal administration in rats. A 0.5% concentration solution of dextran dye suspended in SNOT was administered to OMP-GFP mice through intranasal instillation as described above. Rhodamine dextran (tetramethylrhodamine 3000 MW anionic lysine-fixable dextran) and anionic near-infrared (NIR) dextran in SNOT (Alexa Fluor 680; 3000 MW, Anionic) were used to track the movement of dextran in SNOT in the nasal cavity and olfactory bulb using stereomicroscopy and epifluorescence.

Stereomicroscopy

Mice were euthanized by an overdose of sodium pentobarbital containing euthanasia solution. The dorsal portion of the neurocranium was removed and the olfactory bulb and cerebrum were examined using an Olympus SZX16 research stereomicroscope and photographed using a DP73 cooled digital camera or an Olympus XM10-IR microscope camera. The nose and brain were then immersion-fixed in 10% neutral buffered formalin.

Histopathology

Tissues were fixed in neutral buffered formalin. Noses were decalcified in 13% formic acid and cross-sections were taken at four levels. The four levels of the mouse nose were from the three standard trimming locations[48] with an additional level just behind the third standard location. The fourth level was included because it usually contains more olfactory nerve fibers crossing the cribriform plate than the standard sections. Tissues were processed in a Thermo Scientific Excelsior AS tissue processor. Hematoxylin and eosin–stained sections of lung and nose were evaluated by a board-certified veterinary pathologist.

Plastic Sections

Plastic sections were used to confirm entry of rhodamine dextran into the neuroepithelium of the nose. Lysine fixable dextran dyes are preserved with aldehyde fixatives such as formalin, but the fluorescence is lost in paraffin-embedded tissue sections. However, the fluorescence of aldehyde fixable dyes can be visualized in unstained plastic tissue sections.

Therefore, sections of level T2 of the nose from nine mice exposed to rhodamine dextran in SNOT and two mice exposed to SNOT alone were embedded in plastic using a JB-4 plastic embedding kit as previously described. Suitable-oriented dextran-exposed samples were obtained and photographed from the following time points: 3 hours (n = 2), 6 hours (n = 2), 1 day (n = 3), and 1 week after exposure (n = 1). Plastic sections with adequate orientation from mice exposed to SNOT alone were obtained and photographed from the following time points: 3 hours (n = 1) and 1 week (n = 1) after exposure.

Estimation of Median Diameter From DLS Data

The median of particle diameter was estimated for DLS measurement of particle size because particle diameter distributions tend to be skewed by a small fraction of large particles, thus the median is a better measure of center of the particle diameter distribution than is the mean. In addition, in the nose, large particles can simply be expelled out of the nose with a sneeze or a snort. Conversely, having a large portion of particles small enough to undergo olfactory transport is important in evaluating potential nose-to-brain transport. To estimate the median particle diameter for each DLS data set, the discrete diameter measurements from DLS were repeated to create a new data set comprising the discrete diameter measurements proportional to their average percentage across the trials (rounded up to the next integer). For example, the vesicle diameter measured 32.67 nm was on average 20.39% of the vortexed SNOT, so the newly created data setfor vortexed SNOT sample contained the value 32.67 repeated 21 times. A Wilcoxon rank sum test was then used on each new data set to estimate a (pseudo) median value along with corresponding 95% confidence intervals via the approximation method. To estimate the 1st percentile of each data set, a function was approximated from the original distribution of discrete diameter measurements and used to integrate from the lowest diameter measurement to the 1^{st} percentile. All analyses were conducted using R (v 3.6.0) in RStudio (Vienna, Austria).