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Near-Infrared Fluorescent NanoGUMBOS for Biomedical Imaging

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ABSTRACT Herein, we report on near-infrared (NIR) fluorescent nanoparticles generated from an emergent class of materials we refer to as a Group of Uniform Materials Based on Organic Salts (GUMBOS). GUMBOS are largely frozen ionic liquids, although the concept is more general and is also easily applied to solid ionic materials with melting points in excess of 100 °C. Nanoparticles based on GUMBOS (nanoGUMBOS) derived from a NIR fluorophore are prepared using a reprecipitation method and evaluated for *in vivo* fluorescence imaging. Due to their uniformity, single-step preparation, and composite nature, nanoGUMBOS help to resolve issues with dye leakage problems innate to alternate cellular stains and unlock a myriad of applications for these materials, highlighting exciting possibilities for multifunctional nanoGUMBOS.

KEYWORDS: near-infrared · fluorescence · ionic liquids · GUMBOS · nanoparticles · nanoGUMBOS · biomedical imaging

Near-infrared (NIR) fluorescent materials have been successfully applied in areas such as analyses and sensor development,[1] laser dyes,[2] organic light-emitting diodes (OLEDs),[3] invisible printing inks,[4] photodynamic therapy,[5] and as biomedical imaging contrast agents.[6−10] For *in vivo* imaging applications, the low absorption coefficient of human skin tissues in the 700−1100 nm NIR wavelength region[11] minimizes scattering and background interference, allowing for deep tissue imaging.[12] A number of NIR-emissive materials have been exploited based on their desirable luminescence in this spectrally quiet region, especially those that fall in the nanoregime. These nanomaterials include quantum dots,[13] single-walled carbon nanotubes,[14] lanthanides,[15] fluorescent proteins,[16] gold nanoshells,[12] fluorophore-tagged polymers,[17] and organic dyes.[18,19] However, many of these nanomaterials have had concerns raised regarding both their environmental safety and their cytotoxicity. For instance, quantum dots have been reported to cause microbial toxicity[20] and pose serious environmental safety concerns which are difficult to either control or predict. In this regard, the development of alternative nanomaterials that are biocompatible, nontoxic, and tunable, while exhibiting well-defined delivery behavior, is highly sought.

When employed for biological applications such as imaging, fluorophores are typically encapsulated or doped into a polymeric[21] or silica[22] carrier particle, primarily for purposes of biocompatibility. However, dye encapsulation using these materials often leads to additional challenges such as dye leakage[21] and permeability problems.[23] There are also concerns that the use of surfactant stabilizers in the preparation of these particles may induce systemic toxicity.[24] Again, an urgent need remains for the development of uniform, nonleaking, and additive-free luminescent particles for bioimaging.

Within our laboratories, we have recently developed an emergent class of highly promising nanomaterials we will collectively refer to as GUMBOS (**G**roup of **U**niform **M**aterials **B**ased on **O**rganic **S**alts). A range of stable GUMBOS can be formed in the nanoregime from ionic liquids (ILs) that melt above room temperature. In general, the low melting points of ILs stem from the asymmetry of the component ions and the resultant poor crystal packing,[25] a feature open to design. Of course, nanoGUMBOS developed from ILs enjoy many of the unique properties associated with this class of material, including negligible vapor pressure, variable solubility, nonflammability, high thermal stability, ionic conductivity, and recyclability.[26] In the current application, however, GUMBOS formed from ionic materials that do not comply with the traditional working definition of an IL and melt above 100 °C[27] are also useful

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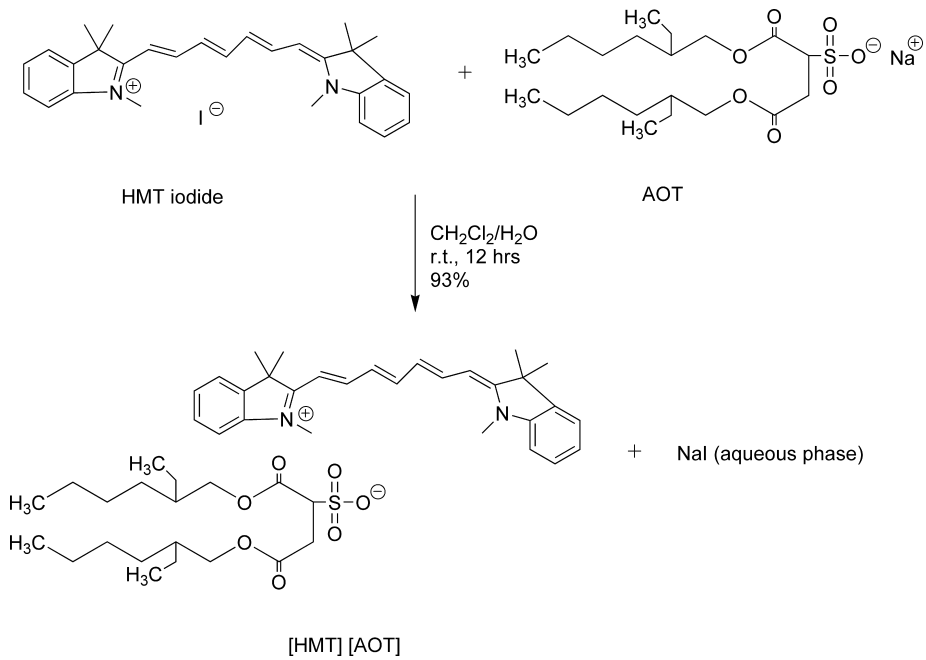
building blocks in GUMBOS formation. The most attractive feature of nanoGUMBOS is the ability to gather within the same nano-object several complementary or orthogonal properties, leading to the prospect of developing multifunctional GUMBOS. This designer characteristic borrows from lessons learned in IL technology, namely, that careful selection and pairing of a functional cation with a dissimilarly functional anion leads to a tailored material displaying bimodal properties.

In this study, we report on a class of fluorescent nanoparticles based on the concept of GUMBOS. These nanoparticles display uniform, stable NIR luminescence suitable for fluorescence imaging applications. As proof of concept, the unique spectral properties of [HMT][AOT] nanoGUMBOS and its performance in cellular imaging studies are summarized in this work. To the best of our knowledge, this is the first report of IL-based NIR fluorescent nanoparticles. Our results suggest that nanoGUMBOS might offer unique possibilities for *in vivo* NIR fluorescence imaging without the need for dye carriers, providing potential as contrast agents in other medical imaging modes, as well.



Scheme 1. Synthesis of [HMT][AOT] by anion exchange reaction.

RESULTS AND DISCUSSION

Synthesis, Characterization, and Optical Properties of NIR GUMBOS and NanoGUMBOS. The GUMBOS reported in this article were produced from a cationic NIR core unit following a modification of anion exchange procedures reported elsewhere.[28−30] We selected for this study a model cationic NIR-emitting cyanine dye 1,1′,3,3,3′,3′-hexamethylindotricarbocyanine (HMT) iodide. We note that cyanine dyes such as indocyanine green (commonly known as cardiogreen) are approved by the FDA for use in bioimaging in coronary angiography, evaluation of hepatic function, and monitoring blood flow.[31−34] For studies involving human cells or animal models, more careful choices of nontoxic NIR dyes are of paramount importance.

An example of an anion exchange reaction between HMT iodide and sodium bis(2-ethylhexyl)sulfosuccinate (AOT) is shown in Scheme 1. We note that AOT has been previously used as anion to make ILs.[35] The various anions used in the formulation of our GUMBOS were selected for their varying hydrophobicities, geometries, and masses. All GUMBOS obtained were characterized by nuclear magnetic resonance (NMR) and elemental microanalysis and yielded results consistent with expectations, and the data are provided in the Supporting Information (Figure S1, Tables S1 and S2).

As expected, the resulting NIR-emitting GUMBOS displayed variable physical properties (melting point, solubility) dependent upon variations in the anion (Table S1). This illustrates the tunability of our GUMBOS, allowing the design of a variety of physicochemical properties targeting select applications. In general, we found that the melting point decreased with an increase in the size of the anion for borate-containing anions. For instance, HMT with a relatively smaller tetrafluoroborate anion had a melting point of 175 °C, whereas the melting point decreased to 95 °C when a larger 3,5-bis(trifluoromethyl)phenyltrifluoroborate anion was used (Table S1). This observation is consistent with that of Larsen and co-workers who investigated the factors that affect melting point for imidazolium ILs.[36] The authors concluded that the reduced crystal packing efficiency resulting from large anion incorporation played a profound role in depressing the melting point. Additionally, alkylation of the anion further frustrated the ion packing, causing a further drop in the melting point.[36] It is worth noting that databanks and semiempirical models to predict melting points of ILs have been published.[37] References such as these serve as valuable resources when considering the design and synthesis of future GUMBOS.

The solubility of ILs in water has been shown to be highly dependent on the choice of the anion.[38] As expected, the miscibility of the various GUMBOS with water was also highly variable and dependent on the anion used in GUMBOS formation. The water-immiscible GUMBOS are suggestive of poor hydrogen bonding interactions, as exemplified by [HMT][AOT]. However,

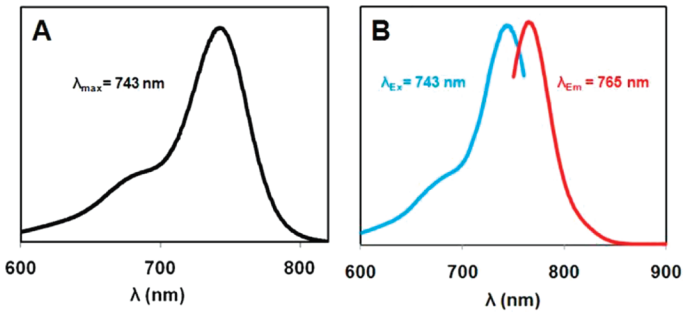


Figure 1. (A) Absorbance profile and (B) fluorescence excitation and emission spectra for 1.0 μM [HMT][AOT] in ethanol; λ_{ex} = 743 nm, λ_{em} = 765 nm.

some GUMBOS based on relatively hydrophilic iodide and boron-containing anions such as tetrafluoroborate showed enhanced aqueous solubility, possibly a consequence of the additional hydrogen bonding afforded by the degree of fluorination. In addition, the electron-withdrawing properties of fluorine may induce dipole moments, resulting in dipole-induced dipole interactions with the surrounding water molecules.[39] This postulate is supported by water immiscibility of HMT containing the 3,5-bis(trifluoromethyl)phenyltrifluoroborate anion, which may be attributed to a reduced net dipole moment arising from symmetric trifluoromethyl substitution (Table S1).

In each case, solutions of GUMBOS based on the [HMT$^+$] cation were excellent absorbers of NIR irradiation. An example of this observation is shown in Figure 1A for a 1.0 μM ethanolic solution of [HMT][AOT] GUMBOS, where peak absorption occurs at 743 nm. As a result of the parent HMT cation, the GUMBOS fluoresce strongly in the NIR region, peaking near 765 nm, with the fluorescence excitation and emission spectra following the mirror-image rule (Figure 1B) as expected from the Franck−Condon principle. The fluorescence of equimolar [HMT][I] and [Na][AOT] ethanolic mixture was identical to that of [HMT][AOT] GUMBOS ethanolic solution shown in Figure 1B. Unlike in the nanoGUMBOS, the anion does not have pronounced influence on the fluorescence emission of the cationic dye in dilute solution.

In the initial report on the preparation of nanoGUMBOS, we set forth a precedent for employing frozen ILs to manufacture size-controlled nanoparticles.[40] In our earlier study, we developed a "melt−emulsion−quench" approach for the controllable formation of particles with average diameters spanning the 45 to 3000 nm range. In the current work, the preparation of nanoGUMBOS was achieved using a modified reprecipitation method,[41−45] which is simpler to implement and more rapid. In a typical preparation, 100 μL of a 1 mM solution of GUMBOS precursor dissolved in ethanol was rapidly injected into 5 mL of triply deionized water in an ultrasonic bath, followed by further sonication for 2 min. The ethanolic pre-GUMBOS solution and water were both filtered prior to preparation of the nanoparticles using 0.2 μm nylon membrane filters. Postpreparation, the particle suspension was aged for 1 h in the dark. The average particle size and size distribution of the prepared nanoGUMBOS were obtained by use of transmission electron microscopy (TEM) and dynamic light scattering (DLS). We note that most of the water-miscible solvents such as acetonitrile, tetrahydrofuran, dimethyl sulfoxide, *N*-methyl pyrrolidinone, and acetone may be used in reprecipitation to obtain nanoGUMBOS dispersed in water.

The reprecipitation synthetic method yields primarily spherical or slightly ovate nanoparticles as confirmed by TEM. A representative TEM micrograph of nanoGUMBOS with an average particle diameter of 71 ± 16 nm is shown in Figure 2A. The polydispersity index obtained for these samples by use of DLS was generally quite good, usually under 0.100. The average size and size distribution of other nanoGUMBOS is provided in the Supporting Information (Table S1).

The prepared NIR-emitting nanoGUMBOS displayed optical properties which were strikingly different from that of the initial ethanolic dye solution. The absorbance and fluorescence spectra of all the nanoGUMBOS investigated are shown in the Supporting Information (Figure S2). The absorbance spectra for our nanoGUMBOS were generally broad and bimodal, extending to significantly lower wavelengths. For example, the absorbance of the [HMT][AOT] suspension was very broad, spanning from well below 600 nm to well over 800 nm (Figure 2B). When measured at an equivalent absorbance value, the emission intensity for the nanoparticle suspension was of comparable intensity but hypsochromically shifted by roughly 8 nm as compared to the parent compound dissolved in an EtOH solution (Figure 2C). This observation is highly characteristic of dye aggregation,[41,46] in this case reflecting intermolecular interactions between [HMT$^+$] units. The deconvoluted spectral properties of the nanoGUMBOS suggest that both H- and J-aggregates form in different proportions. The predominant aggregates formed seem dependent on the structure of the anion resulting in different arrangements of molecules in the nanoGUMBOS.

Decreased fluorescence intensity has also been observed for highly crystalline systems as a result of enhanced internal conversion processes.[41,46] However, we can essentially rule out this possibility as powder X-ray diffraction measurements (data not shown) of the dried nanoGUMBOS reveal that the particles are in fact predominantly amorphous (see also the SAED pattern inset in Figure 2A). Such X-ray diffraction patterns have been previously used to confirm amorphous nature of nanoparticles.[44]

If the HMT-derived nanoGUMBOS are dried and then redissolved in ethanol, they retain the spectral characteristics of the parent solution (Figure 3). This observation supports our contention that the remarkable variations in both absorbance and fluorescence properties wholly arise from the aggregation state of the [HMT$^+$]

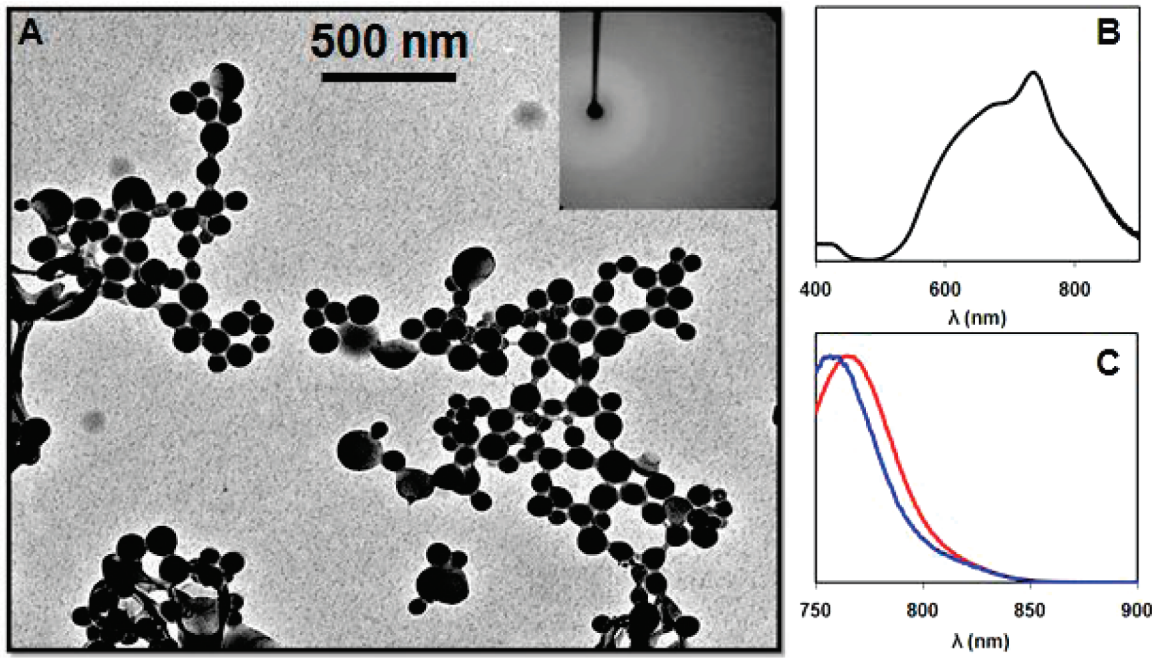


Figure 2. (A) TEM micrograph of aqueous [HMT][AOT] fluorescent NIR nanoGUMBOS with an average diameter near 71 ± 16 nm; the inset shows a selected area electron diffraction pattern (SAED) for the [HMT][AOT] GUMBOS. (B) Absorbance spectrum of the [HMT][AOT] nanoparticles illustrated in panel A, and (C) comparison between the normalized fluorescence emission spectrum of the freely dissolved [HMT][AOT] IL (1.0 μM in ethanol; red profile) and [HMT][AOT] nanoGUMBOS (blue profile) for matched absorptivity at the excitation wavelength (λ_{ex} = 743 nm).

within the nanoGUMBOS material. This also demonstrates that the intrinsic spectroscopic properties and chemical identity of the GUMBOS precursor ("monomer" ion units) remain unaltered during nanoparticle formation. This observation is important when considering the potential of GUMBOS for use as drug delivery and therapeutic vehicles.

Cellular Uptake of NanoGUMBOS. In order to demonstrate the potential of nanoGUMBOS as contrast agents for biomedical imaging applications, we examined cellular uptake and fluorescence images of these particles using monkey kidney fibroblast (Vero) cells. Vero cells have previously been used for screening *Escherichia coli* toxin and can serve as host cells for viruses as well as eukaryotic parasites. In the present study, Vero cells were used as model cells to investigate the cellular uptake of our nanoGUMBOS. Using an epifluorescence microscope, we captured fluorescence images revealing that [HMT][AOT] nanoGUMBOS can be internalized and subsequently visualized within viable Vero cells after 24 h of incubation (Figure 4). These studies suggest the exciting potential of using nanoGUMBOS in cellular imaging. The apparent homogeneous fluorescence suggests that the nanoGUMBOS distribute nonspecifically inside the cells and are primarily located within the cytoplasm. The uptake of these nanoparticles is presumably due to the well-known adsorptive endocytosis process previously demonstrated for mesoporous silica nanoparticles;[47] however, the exact cell-penetrating mechanism and localization of the nanoparticles is currently under investigation.

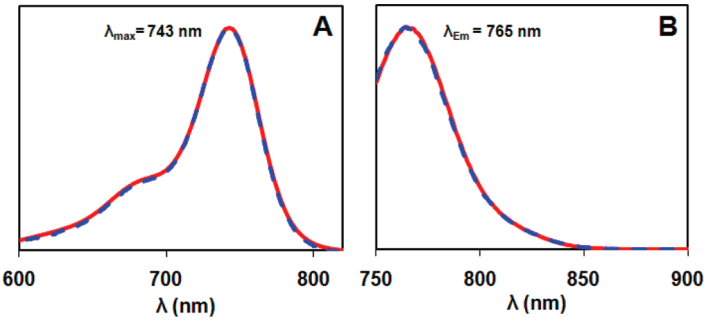


Figure 3. Normalized (A) absorbance and (B) fluorescence emission spectra of [HMT][AOT] (1.0 μM solution in EtOH; solid curve) and GUMBOS from Figure 2A redissolved in EtOH (dashed curve).

CONCLUSIONS

In summary, we have synthesized and investigated preliminary spectral and cellular uptake properties of a series of NIR fluorescent nanoscale ionic materials we term nanoGUMBOS. By appropriate selection of a cationic NIR dye, these fluorescent nanoparticles were designed to exhibit absorbance and luminescent properties in the tissue-accessible NIR region of the electromagnetic spectrum. In this study, nanoGUMBOS 71 ± 16 nm in diameter were fabricated by use of a simple, rapid, repeatable, and additive-free reprecipitation method requiring neither special nor costly lab apparatus. Beyond the ease of preparation, this work pre-

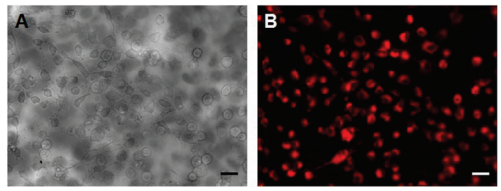


Figure 4. Cellular uptake studies using a monkey kidney fibroblast (Vero) cell line. (A) Phase contrast micrograph and (B) corresponding fluorescence image of Vero cells incubated for 24 h with 8.0 μg mL^{-1} [HMT][AOT] nanoGUMBOS. The fluorescence was collected using a propidium iodide (PI) filter set: λ_{ex} = 540 nm; λ_{em} = 617 nm long pass. Scale bars are 10 μm.

sents an entirely nonconventional direction for preparing contrast agent nanoparticles directly from tailored ionic materials. Our approach alleviates current problems associated with dye leakage and other challenges encountered in dye encapsulation, as well obviating intensive purification steps required when surfactants are used to prepare dye nanoparticles. It is also noteworthy that the spectral properties of our GUMBOS deviate markedly from the freely dissolved dye present in solution. Thus, the possibility for tuning the optical properties of GUMBOS by variation in the parent ions offers a unique and exciting advantage for these nanomaterials. It was also demonstrated that these NIR fluorescent nanoparticles could be efficiently taken up *in vitro* by Vero cells, suggesting intriguing potential for noninvasive biomedical imaging. Even more exciting is the prospect of tailoring nanoGUMBOS for delivery into designated cellular structures. In any case, examination of the data presented for these cellular studies highlights a new potential for biomedical imaging using NIR fluorescent nanoGUMBOS.

It should be possible to extend the findings from this investigation to fashion GUMBOS combining diverse functionalities (*e.g.*, redox, superparamagnetic, luminescent, thermochromic, ligating) in order to arrive at truly multifunctional hybrid materials. One could easily envision future polyfunctional nanoGUMBOS with applications including targeted diagnosis and therapy, photothermal cancer therapy, scintillators, radiosensitizers, biological separations, solar cells, and materials with defense applications (*e.g.*, microwave absorbers, security barcodes). The incorporation of high atomic number elements such as iodine or gold may enable their use as contrast agents for X-ray computed tomography (CT) scans. Likewise, GUMBOS carrying gadolinium chelates may open up potential for clinical diagnosis by use of magnetic resonance imaging (MRI).

MATERIALS AND METHODS

Materials. 1,1′,3,3,3′,3′-Hexamethylindotricarbocyanine (HMT) iodide (97%), bis(2-ethylhexyl)sulfosuccinate (AOT) sodium salt (≥99%), sodium tetrafluoroborate (≥98%), potassium 3,5-bis(trifluoromethyl)phenyltrifluoroborate, lithium bis(trifluoromethane)sulfonimide (99.95%), and ethanol (spectroscopic grade) were purchased from Sigma Aldrich and used as received. Triply deionized water (18.2 MΩ · cm) from an Elga model PURELAB ultra water filtration system was used for all preparations of the NIR GUMBOS and nanoGUMBOS. A BRANSON 3510R-DTH model bath ultrasonicator (335 W, 40 kHz frequency) at room temperature was used in the preparation of nanoGUMBOS. Vero cells were obtained from the School of Veterinary Medicine (Louisiana State University, Baton Rouge, LA). Carbon-coated copper grids (CF400-Cu, Electron Microscopy Sciences, Hatfield, PA) were used for TEM imaging.

Synthesis and Characterization of NIR GUMBOS. The NIR GUMBOS (mostly ILs) were prepared using anion exchange procedures similar to those reported in the literature.[25−27] The synthesis of 1,1′,3,3,3′,3′-hexamethylindotricarbocyanine bis(2-ethylhexyl)sulfosuccinate ([HMT][AOT]) is described as a representative procedure. An amount of 30 mg (0.056 mmol) of 1,1′,3,3,3′,3′-hexamethylindotricarbocyanine (HMT) iodide and 24.86 mg (0.056 mmol) of sodium bis(2-ethylhexyl)sulfosuccinate (AOT) salt was dissolved in a mixture of methylene chloride and water (2:1 v/v) and allowed to stir for 12 h at room temperature (Scheme 1). The methylene chloride bottom layer was washed several times with water, and the product was obtained from the organic lower layer and dried by removal of solvent *in vacuo*. Further freeze-drying to remove traces of water afforded 43.02 mg (93% yield) of [HMT][AOT]. All GUMBOS obtained were characterized by ^{1}H NMR (Bruker Avance 400, $CDCl_3$) and elemental microanalysis (Atlantic Microlab, Norcross, GA). In addition, ^{19}F NMR (Bruker DPX 250, $CDCl_3$) was used to confirm anion exchange for fluorine-containing anions. Melting points of the GUMBOS were determined using a MEL-TEMP capillary melting point apparatus.

Synthesis of NIR NanoGUMBOS. The nanoGUMBOS were prepared from GUMBOS using a modified simple, additive-free reprecipitation method similar to that used for organic nanoparticles.[24,41−45] In a typical preparation, 100 μL of a 1 mM solution of GUMBOS precursor dissolved in ethanol was rapidly injected into 5 mL of triply deionized water in an ultrasonic bath, followed by further sonication for 2 min. The ethanol and water were both filtered prior to preparation of the nanoGUMBOS using 0.2 μm nylon membrane filters. Postpreparation, the particle suspension was aged for 1 h in the dark.

Characterization of Size and Morphology of NIR NanoGUMBOS. The average particle size and size distribution of the prepared nanoGUMBOS were obtained by use of transmission electron microscopy (TEM) and dynamic light scattering (DLS). TEM micrographs were obtained using an LVEM5 transmission electron microscope (Delong America, Montreal, Canada). The NIR nanoGUMBOS dispersion (1 μL) was dropcasted onto a carbon-coated copper grid and allowed to dry in air at room temperature before TEM imaging.

X-ray Diffraction Analysis of NIR NanoGUMBOS. X-ray diffraction measurements of dried nanoGUMBOS were obtained on a Nonius Kappa CCD diffractometer by long exposures with Mo Kα radiation and rotation of samples about the vertical axis.

Absorption and Fluorescence Studies of NIR GUMBOS and NanoGUMBOS. Absorbance measurements were performed on a Shimadzu UV-3101PC UV−vis−near-IR scanning spectrometer (Shimadzu, Columbia, MD). Fluorescence emission was collected using a Spex Fluorolog-3 spectrofluorimeter (model FL3-22TAU3); Jobin Yvon, Edison, NJ). A 0.4 cm^2 quartz cuvette (Starna Cells) was used to collect the fluorescence and absorbance against an identical cell filled with water as the blank.

Cellular Uptake Studies of Nano-GUMBOS by Vero Cells. Vero cells (isolated from kidney epithelial cell lining extracted from an African green monkey, *Cercopithecus aethiops*) were used as a model cell line for this study using [HMT][AOT] nanoGUMBOS. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) before studies. The Vero cells were then loaded into an eight well glass plate (2.5 × 10^5 cells/well) and incubated with

HMT AOT nanoparticles (8 μg/mL) for 24 h. The cells are then washed twice with phosphate buffered saline (PBS) and immediately visualized using a Zeiss epifluorescence microscope equipped with a Zeiss digital camera for image acquisition. Negative controls were also prepared by loading the Vero cells into the wells which did not contain dye.

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Supporting Information Available: Yields, melting point, aqueous solubility, NMR spectra (^{1}H, ^{19}F NMR), elemental analysis of HMT-derived GUMBOS; absorbance, fluorescence, and size of HMT-derived nanoGUMBOS. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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