Development of a new generation of sensitive, fluorescence-based nitrate sensors for use in soil and water

Basic Information

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Statement of regional or state water problem:

Throughout the world, eutrophication of tributaries, rivers, estuaries, and coastal marine ecosystems by point and non-point sources (e.g. industry, sewage, development, atmospheric deposition and agriculture) is changing these ecosystems' biogeochemical function, ecology, and the human institutions dependent on them [1, 2]. Roughly one-third of the nitrogen (N) reaching Long Island Sound (LIS) is derived from non-point sources, and these non-point sources, and the sinks, transport and processing of N in pathways leading to LIS, are not well understood. Integrating data from 28 water monitoring stations (23 in CT) with land-use/land-cover information, population density, runoff volumes, and other landscape characters, Mullaney et al. (2002) developed a simple linear regression model capable of predicting N-loads and yields in streams as a function of watershed characteristics [3]. This model was used to estimate N-loading in unmonitored watersheds, but unexplained discrepancies were found that indicated unknown but important system drivers were affecting N-loading in both agricultural and forested landscapes [3]. In particular for the current effort, researchers noted that the type of forest cover (e.g. percent deciduous tree cover) significantly affects N-loading [3], suggesting either that the dominant tree types themselves [4], and/or perhaps edaphic factors correlated with those forest communities [5], strongly affect N-yield from forested areas. Recently published data suggest that dominant northeastern forest deciduous and evergreen species are associated with distinct rates of N-cycling and N-retention in the soils supporting them [4, 6]; these distinct N-cycling rates could lead to distinct N-load "signatures" in watersheds that correlate with forest cover.

Ultimately, we would like to explore how patterns of N-cycling, and dynamics of various dissolved N pools, correlate with the patterns and growth of diverse New England forests, but current monitoring methods are inadequate. Though current ecosystems ecological techniques for quantifying net and gross mineralization and nitrification, as well as microbial immobilization, of nitrogen have certainly led to greater understanding of patterns in and mechanisms underlying N-cycling in forested systems, those techniques invariably require destructive harvest of soil prior to assay [7]. Unfortunately, digging up the belowground system under study necessarily leads to severing plant roots that contribute carbon to soil, breaking hyphae of mycorrhizal and saprotrophic fungi, mixing up soil layers, and breaking up soil aggregates that otherwise can have cores hypoxic enough to support denitrification. Ecosystems ecology sorely needs a suite of miniature sensors capable of being deployed to continuously monitor dissolved nitrogen species in soils. Because soil processes are notoriously heterogeneous, ideal would be development of an inexpensive and sensitive enough design to support deployment of a suite of such sensors in multiple locations across a watershed so that networked, continuous pool data could be gathered across the landscape. Ecosystems ecologists have long used stream water concentrations of ions as an integrated measure of terrestrial system output (for example demonstrating that immobilization of otherwise mobile essential nutrients in new aggrading forest biomass after logging leads to dramatic and seasonal decreases in nitrate concentrations in streamwater [8]). But the N processing on the terrestrial landscape itself has been studied destructively, not continuously, and often not *in situ*.

Ecosystems scientists are not alone in needing improved monitoring tools; land managers and environmental engineers also seek sensors that can be deployed in order to detect plumes of contaminants, including nitrate, moving through groundwater. Development of small nitrate sensors is already underway in that context, e.g. at UCLA (see the Center for Embedded Networked Sensing focus on contaminants and terrestrial ecosystems [9]). However, the concentrations of nitrate in a contaminated plume are much higher than background concentrations in forested systems. McDowell showed that nitrate concentrations in soil solution (extracted using zero tension lysimeters, and analyzed in the lab) were between 0 and 1 mg L^{-1} over a time span of ten years in unmanipulated hardwood and pine stands at Harvard Forest [10]. In contrast, in plots experiencing chronic high N deposition (15 g N m^{-2} yr^{-1}) for those same ten years, nitrate concentrations in soil solution ranged between 10 and 28 mg L^{-1} in pine and hardwood stands. In order to have any hope of monitoring, continuously, shifts in pools of nitrate (and potentially, ultimately, other dissolved N species) in soil solution under particular forest tree species, and in streams draining relatively pristine rather than highly polluted watersheds, very sensitive, miniature sensors are needed. Ideally, such a sensor would be inexpensive

and could be coupled into some of the already established protocols for networking sensors like those being developed at CENS. The first step, however, is how to improve sensitivity of sensors to nitrate in low (background or pristine) concentration in freshwaters and in soil solution, and how to package the sensors in a miniaturized form that is relatively inexpensive.
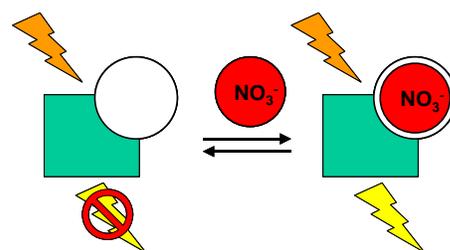
We aim to develop miniature, "turn-on", fluorescence sensors for nitrate that, ultimately, can be deployed in sets in soil and in freshwaters to report nitrate concentrations continuously in background and low contamination ranges – e.g. 0-2 mg per liter. Nitrate sensors for deployment *in situ* are already on the market, for example designed for work in oceans (e.g. UV sensor, Satlantic's ISUS V2) and streams and groundwater (e.g. Hydrolab nitrate ion selective electrodes [11]). Some are already very sensitive. Satlantic, for example, claims sensitivity of 0.007-28 mg nitrate per liter, +/- 0.028 or 10% of the measurement, whichever is larger. But, the sensor is 2 ft long, 4 inches in diameter, and weighs 11 lbs. New amperometry-based nitrate detectors for use in soil and groundwater are becoming more sensitive, but to date their major focal application has been on highly contaminated nitrate plumes in groundwater [9], though the focus is also shifting to detection in more undisturbed, terrestrial ecosystems. It is important to understand the background N-cycling processes occurring in more pristine environments in order to understand the magnitudes and multiple mechanisms of human impacts on that cycling, yet we lack the tools for *in situ* monitoring of pools within the heterogeneous N-cycling pool and flux network.

Significance: Statement of results or benefits

Fluorescent sensors are an attractive target for developing a next generation of nitrate detection systems, because fluorescence methodologies are more sensitive, and easier to apply than current technologies. Assistant professor of chemistry Burdette specializes in the chemical synthesis and the principles of photochemistry relevant to sensor construction. Funding from CTIWR for 2 years that is earmarked to support work on developing a fluorescence-based nitrate sensor, and would provide the necessary seed money to initiate the molecular design of a sensing system. Cardon can guide the preliminary tests of sensitivity in real samples from the field, and analysis of comparable data from other detection methods (e.g. anion exchange membrane and buried resin bag-estimates, as well as KCl extraction-estimates of nitrate pools in soil [7]). With a proof-of-concept sensor in hand, more funding (e.g. from NSF's Bioengineering and Environmental Systems program) will be sought to advance the technology beyond molecular design toward the miniaturization and field-readiness of continuous sensing systems. Because Cardon serves on the national SAMSI program steering committee for development of mathematical and statistical analysis of sensor network data [12], she already has the necessary contacts to help bring the established technology rapidly to an interested community nationwide. Cardon has already collaborated with John Mullaney at USGS and Paul Stacey at DEP in CT writing grant proposals (to NSF) to further explore nitrogen processing in Connecticut's watersheds and Long Island Sound. The links from the sensor development proposed here to CT government agencies thus promises to be a natural progression.


Figure 1. Representation of a typical fluorescent sensor. Whether sensor refers to a small molecule, a polymer or a device, the signaling action involves fluorescence emission (yellow bolt) that occurs only in the presence of analyte binding (NO_3^-) when the sensor is exposed to excitation light (orange bolt).

Nature, scope, and objectives of the project

Fluorescent Sensors (Fig. 1). Fluorescent sensors have been vital in identifying the cellular functions of metal ions [13], as well as the impact of environmental contaminants like mercury [14] and polychlorinated biphenyls [15]. With appropriate molecular design, fluorescent sensors are capable of making sensitive measurements using signals that are easy to monitor [16]. Since these chemical tools are typically constructed using modular approaches, simple structural modifications can be made to adapt the sensor for a variety of different applications and circumstances. Analyte concentrations from sub-pM (equivalent of 0.1 µg/L of nitrate) to saturated solutions can be accurately measured. In addition to these advantages, fluorescent sensors

are particularly amenable for monitoring environmental analytes because the imaging reagents and instrumental techniques are non-evasive and simple to employ. One distinct improvement over conventional techniques for measuring nitrate concentrations is that sensors are typically inexpensive and can be designed to be either disposable or reusable. As a result, an array of many individual sensors can be distributed easily over a large area and monitored by one research worker using an inexpensive, portable fluorescence spectrometer. While methodologies for constructing some types of fluorescent sensors are straightforward, designing useful sensors for anions presents a significant challenge. Although fluorescent anion sensors exist, very few systems have been reported for nitrate [17].

Figure 2. The structure of the nitrate anion. The structure on the right shows the composite resonance structure the best describes the chemistry and structure. The molecule is trigonal planar (120° O-N-O angles) and has a negative charge distributed evenly over the 3 oxygen atoms.

Nitrate chemistry. The most challenging obstacle to overcome when constructing a nitrate sensor is finding a receptor to bind what is essentially a non-coordinating anion (Fig. 2). Nitrate is a weak base that does not form covalent bonds with metal cations or protons readily, a behavior that is consistent with it being the conjugate base of a strong acid (nitric acid). In the majority of nitrate complexes characterized crystallographically to date, the nitrate group is located several angstroms away from other atoms, hence the nomenclature "non-coordinating" [18]. Unlike metal cations that can form strong covalent bonds with receptors containing electron-donating atoms like oxygen or nitrogen, anion receptors usually rely on noncovalent interactions like hydrogen bonding or electrostatic interactions for chelation [19]. Noncovalent interactions are weak forces, so it is counterintuitive that a receptor using these interactions can bind an analyte tightly; however, if a receptor takes advantage of several noncovalent interactions simultaneously, a reasonably high affinity interaction can be established.

Another challenge in anion receptor design is the ability to discriminate between other anions. In particular for nitrate found in the environment, anions like chloride (Cl^-), sulfate (SO_4^{2-}), and phosphate (PO_4^{3-}) could interfere with measurements of nitrate concentration if the receptor lacks selectivity. In order to enhance selectivity for nitrate, the receptor can take advantage of the coordination number and charge density. Nitrate is a trigonal planar anion, as opposed to primary oxoanions competitors like PO_4^{3-} and SO_4^{2-} (tetrahedral), and anions like hydroxide (HO-) and halides (F^-, Cl^-, Br^-, I^-) with simple geometrical shapes. Nitrate is monoanionic, with the charge distributed evenly over the 3 oxygen atoms, making them the primary targets for noncovalent interactions with receptors. The monoanionic, trigonal planar structure of nitrate is an uncommon structural motif for anions found in soil and water. Therefore, receptors that can only accommodate a trigonal anionic guest will provide the desired binding selectivity.

Dendrimers. Dendrimers are globular polymers frequently prepared using conventional organic synthetic methodologies (Fig. 3). When convergent synthetic methods are employed [20], dendrimers have uniform size, shape and molecular weight, a property reminiscent of enzyme structures [21, 22]. Because of their unique properties, dendrimers are attractive targets for a variety of applications in catalysis [23], medicine
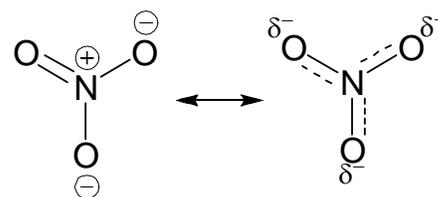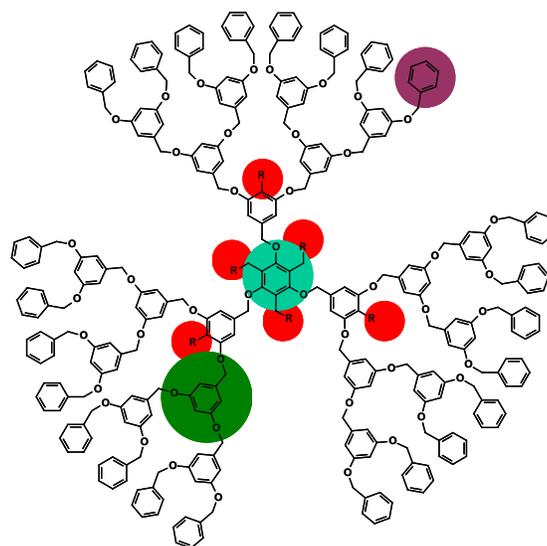
Figure 3. The basic structure of a dendrimer, shown is a 3rd generation poly(benzyl ether). The generation number refers to the number of repeating building blocks (green) between the core (blue) and the periphery (purple). The structure of the repeats and periphery groups determine the physical properties of the dendrimer, and can be changed to attain the desired features. Dendrimers of generation 1-5 are straightforward to prepare. In addition, functional groups such as nitrate receptors or fluorophores (red, "R" groups) can be incorporated at the core, or with in the branching units as needed. For use in water, the peripheral groups will be water soluble (polyethylene glycol) for soil-based systems, hydrophobic organic groups will be utilized.
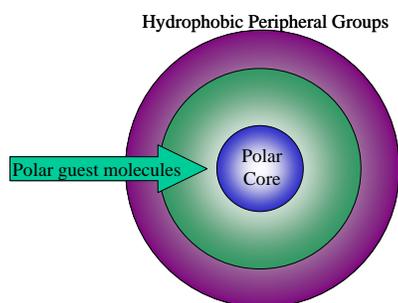
Figure 4. Dendrimers as concentrators. In more hydrophobic environments like soil, a concentrator effect will help to transport nitrate inside the sensor.

[24] and sensing [25]. Several properties of dendrimers make them attractive scaffolds for environmental nitrate sensors. Dendrimers can be prepared with robust chemical linkages that will resist degradation by microrganisms and variations in acidity/basicity in soil and water. Dendrimers also provide encapsulation, and hence protection, for the nitrate receptors and fluorophores that are susceptible to decomposition reactions under the conditions the sensors will be deployed. Most importantly, however, dendrimers can act as "molecule concentrators" (Fig. 4) [26]. When mobility of an analyte like nitrate is lower (like in soil before reaching water), the ability of the dendrimer to concentrate a substrate will amplify the resulting fluorescent signal. The ability of a dendrimer to act as a concentrator, as well as many other physical properties, is dictated by its chemical structure. Dendrimers have three important structural components, the core, the branching groups (polymer repeat units) and the peripheral groups, that can be varied to tune the properties of the resulting macromolecule. As shown in Figure 4, a dendrimer with a hydrophobic periphery and a polar interior will be predisposed to concentrate a charged molecule like nitrate from nonaqueous sources.

Specific Aims
1. Construct nitrate receptors for incorporation into dendritic fluorescent sensors
2. Verify the ability of dendrimers to concentrate nitrate anions
3. Investigate the strategy of using accumulation of anionic charge inside a dendrimer to modulate the emission intensity of polarity sensitive fluorophores
4. Investigate the viability of displacing a negatively charge fluorescence quencher at the core of a fluorescent dendrimer as a detection strategy
5. Investigate the strategy of using charge-induced swelling and contracting of dendrimers to induce fluorescence resonance energy transfer
6. Demonstrate proof-of-concept nitrate sensing with dendrimers in prepared solutions and environmental water samples
7. Devise methodology to attach/adsorb dendritic sensors onto glass surfaces for nitrate sensing in soil samples
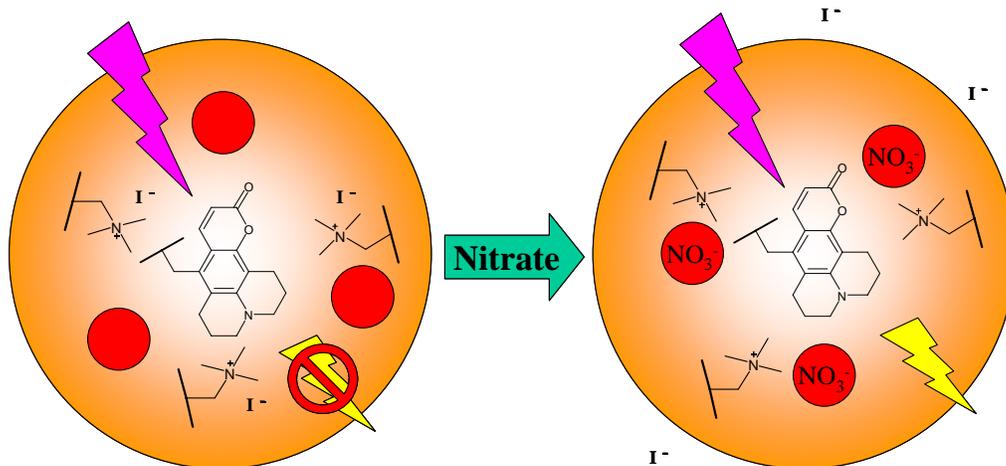
Methodology

General sensor design strategy: dendrimer structure and nitrate receptors. The majority of the existing sensors for anions (e.g. F^-, Cl^-, Br^-, PO_4^{3-}, CO_3^{2-}) rely on either photoinduced electron-transfer (PET), which requires the formation of covalent bonds with the receptor, or electronic energy transfer (EET), a "turn-off" mechanism which requires an analyte with an electronic structure that triggers fluorescence quenching, as the signaling mechanism [17]. Neither of these common strategies is applicative to the disparate requirements of sensing nitrate. An alternative approach to anion sensing is to couple an accumulation of negative charge with a change in the fluorescence intensity. In aqueous solution, nitrate can freely flow into the dendrimer, and be trapped by the receptors; however, in soil nitrate may be less modile with respect to entering the dendrimer. In order to concentrate anions, dendritic molecules will be utilized as concentrators. As a consequence of dendrimers possessing hydrophobic peripheries and polar cores, a concentration gradient is established between the interior of the macromolecule and the exterior that provides the driving force to amass nitrate. All three sensor strategies described below will take advantage of
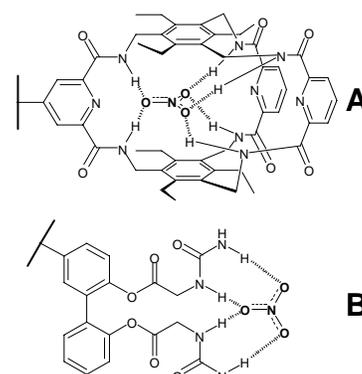
Figure 5. Examples of nitrate receptors. Both cryptate (A) and "tweezer" type ligands selectively bind nitrate (illustrated) in the presence of other anions. The dendrimer attachment site is shown as the squiggly line on the left of each receptor.

the concentrator effect to sequester nitrate in soil. For sensing in water, the hydrophobic peripheral groups will be substituted for with water-soluble groups like short polyethylene glycol (PEG). In addition to concentrator effects, the interior of the dendrimer will be functionalized with molecules capable of binding nitrate to provide anion selectivity and trapping. Several receptors have successfully been applied to nitrate binding (Fig. 5). Both cryptate [27] and "tweezer" type ligands [28] possess nitrate selectivity, because of the common feature that the binding cavity orients hydrogen-bonding interactions in a trigonal planar coordination sphere. These molecules will be the primary candidates for incorporation into the

Figure 6. Mechanism of signaling action with quenching anions. The presence of iodide anions quenches the emission of the coumarin fluorophore (center), when nitrate binds to the receptors, the iodide will be expelled from the dendrimer restoring the emission.

sensors in this initial phase of the research project. Design of new and improved nitrate receptors will not be a major component of CTIWR funded research; however, future proposals and projects will address any limitations of these systems.

Displacement of a quenching anion by nitrate as a sensing strategy. Although nitrate typically is incapable of quenching fluorescence, large anions (e.g. Br^-, I^-) quench fluorescence through enhancement of spin orbit coupling (SOC) [29] and electron deficient anions (e.g. 4-nitrobenzene-sulfonate) through SOC [30] or EET [17]. A simple proof-of-principle sensor for this strategy can be constructed with a dendrimer functionalized with ammonium iodide groups and fluorophores. Diffusion of nitrate into the interior will displace the quenching anions restoring fluorescence (Fig 6).

Principle Findings: Progress Report
General progress. Our work has focused on proof-of-concept research that is focused on two main areas: 1) synthesis of water soluble dendrimers; and 2) synthesis and characterization of sensors containing an anion receptor, a fluorophore and dendrimer mimetic substituents. We have enlisted an undergraduate researcher to continue work this summer. Future progress will be dependent on finding new personnel and additional mechanisms of financial support.

Dendrimers and Water Soluble Dendrimer synthesis. During our first year we synthesize poly(benzyl ether) dendrimers, and to date we have prepared G_1-G_3 dendrimers with
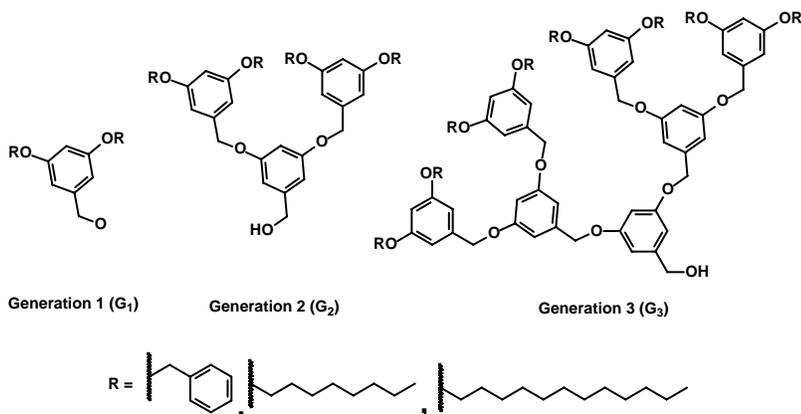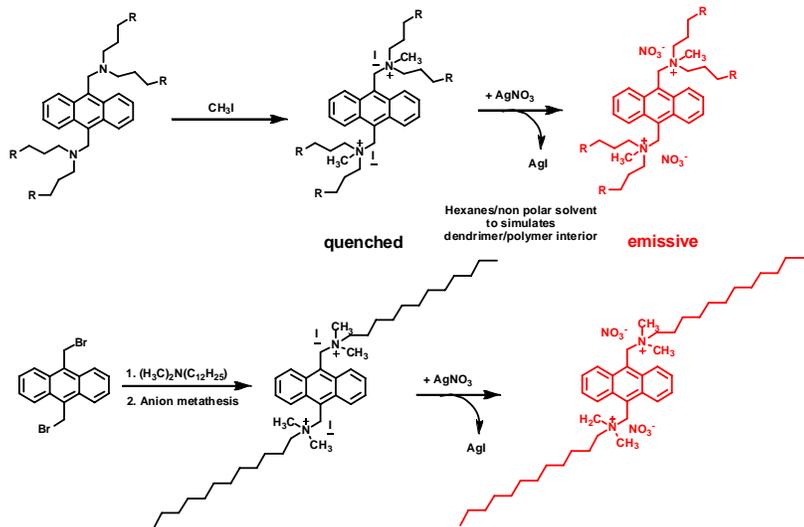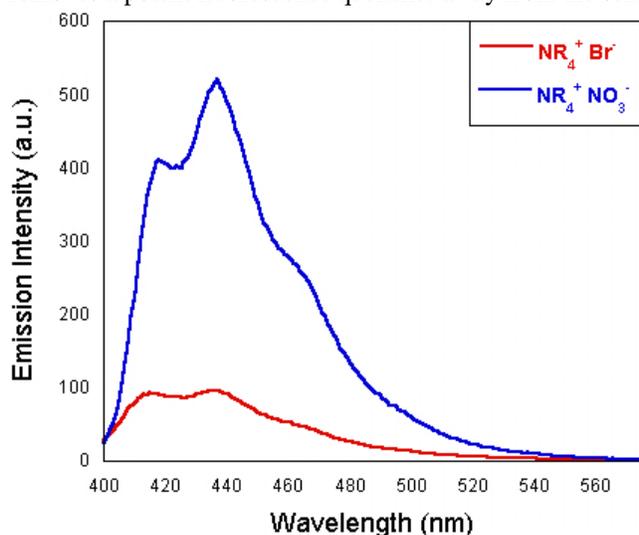

Figure 7. Dendrimers prepared to date
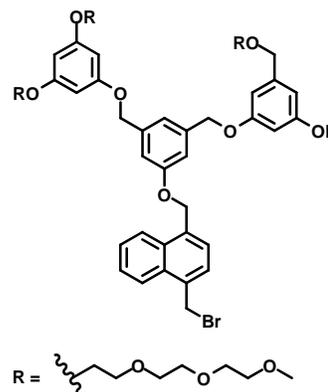
Figure 8. G2 water soluble dendrimer. G1 and G3 versions have also been prepared for incorporation into sensors.

benzyl groups as well as alkyl chains on the periphery (Fig. 7). These components of the model nitrate sensor are available for assembly of the dendritic nitrate sensors proposed originally. We have expanded our efforts in anticipation of constructing sensors for deployment in aqueous solution. Shown in Figure 8 is a water soluble benzyl ether dendron with a naphthalene group at the focal point. The poly(ethylene glycol) units will impart water solubility to future sensors and the naphthalene group is an emissive species that will provide the sensor readout.
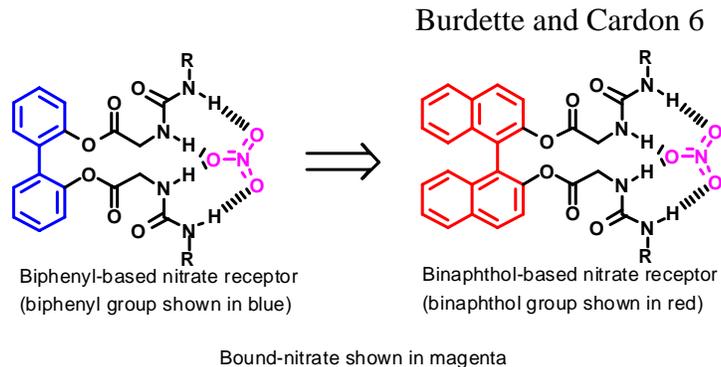
Ntrate receptor synthesis. As we suggested in our

Biphenyl-based nitrate receptor
(biphenyl group shown in blue)

Binaphthol-based nitrate receptor
(binaphthol group shown in red)

Bound-nitrate shown in magenta

Figure 9. We have substituted a fluorescent binaphthol group (red) for biphenyl one (blue) in a nitrate (magenta) receptor. Additional structural changes are possible using our synthesis, and fluorescence studies are being initiated on the new receptor.

original proposal, our 1st generation of nitrate sensors will utilize known nitrate receptors that we will modified to act as fluorescence switches or to contain synthetic handles for attachment to macromolecules. Based on literature precedence, we have modified the structure of a biphenyl-based nitrate receptor to contain a binaphthol scaffold (Fig. 9). We accessed the binaphthol ligand through a multi-step synthesis that is amenable to making a variety of related derivatives or making additional structural modifications. Unlike biphenyl, binaphthol is inherently fluorescent, but we have not observed any emission changes when it binds nitrate; however, this may still be a useful sensor component by incorporating components of the anion sensing mechanism described below.

Investigation of sensing mechanisms. One of the key preliminary studies we have undertaken is an attempt to determine a mechanism that will allow changes in nitrate concentration to increase sensor emission. As described in the proposal, nitrate in a weakly coordinating anion, and therefore many common sensing mechanisms are not appropriate for our purposes. We successfully tested the hypothesis that nitrate can displace a quenching group that is in proximity to a fluorophore by preparing an anthracene derivatives containing two tetra-alkyl ammonium iodides (Fig. 10). The lipophilic alkyl groups to give the resulting sensor solubility in nonpolar solvents like hexanes or toluene. In these nonpolar solvents, the iodide anions form a close ion pair with the

Figure 10. Proof-of-concept nitrate sensing with alkyl ammonium anthracene compounds. Displacement of iodide anions with nitrate anions removes a potent fluorescence quencher away from the sensor.

Figure 11. Fluorescence response of ammonium iodide species to silver nitrate. The nitrate species is approximately 5X more emissive than the iodide consistent with our predictions.

ammonium cation, and the close proximity of the iodide quenches the emission of the anthracene by creating spin-orbit coupling quenching pathways. Subsequent displacement of the quenching iodides by with nitrate restores anthracene fluorescence (Figure 11). The formation of insoluble silver iodide drives the formation of the ammonium nitrate species. we will begin to integrate our sensing strategy with the receptor and dendrimer components. An sample target we are working toward is shown in Figure 12.
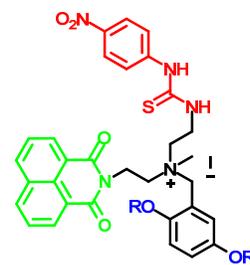
Figure 12. Modular representative of nitrate sensors under preparation. The sensor incorporates dendrimer (blue), anion receptor (red) and a fluorophore (green).

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