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Turn-On Near-Infrared Fluorescent Sensor for Selectively Imaging Serotonin

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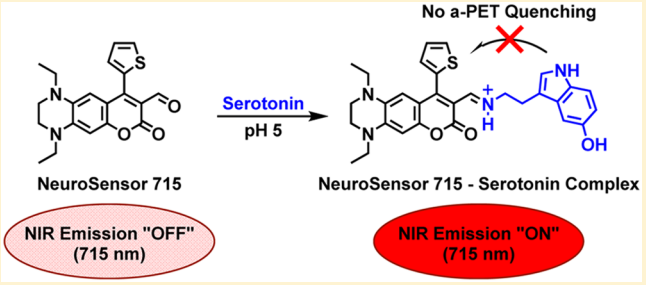
Supporting Information

ABSTRACT: A molecular imaging tool that provides for the direct visualization of serotonin would significantly aid in the investigation of neuropsychiatric disorders that are attributed to its neuronal dysregulation. Here, the design, synthesis, and evaluation of NeuroSensor 715 (NS715) is presented. NS715 is the first molecular sensor that exhibits a turn-on near-infrared fluorescence response toward serotonin. Density functional theory calculations facilitated the design of a fluorophore based on a coumarin-3-aldehyde scaffold that derives from an electron-rich 1,2,3,4-tetrahydroquinoxaline framework, which provides appropriate energetics to prevent the hydroxyindole moiety of serotonin from quenching its fluorescence emission. Spectroscopic studies revealed that NS715 produces an 8-fold fluorescence enhancement toward serotonin with an emission maximum at 715 nm. Accompanying binding studies indicated NS715 displays a 19-fold selective affinity for serotonin and a modest affinity for catecholamines over other primary-amine neurotransmitters. The utility of NS715 toward neuroimaging applications was validated by selectively labeling and directly imaging norepinephrine within secretory vesicles using live chromaffin cells, which serve as a model system for specialized neurons that synthesize, package, and release only a single, unique type of neurotransmitter. In addition, NS715 effectively differentiated between cell populations that express distinct neurotransmitter phenotypes.



KEYWORDS: *fluorescent sensor, near-infrared, live cell imaging, molecular imaging, neurotransmitter, serotonin*

Serotonin (5-hydroxytryptamine) is a critical monoamine neurotransmitter in the central nervous system that plays a key role in regulating emotion, mood, and sense of well-being.[1] Serotonergic neurons synthesize, store, transport, and release serotonin analogous to other specialized neurons that govern only a single, unique type of monoamine neurotransmitter. The biosynthesis of serotonin transpires within the cell bodies of serotonergic neurons, which are localized to discrete cell clusters within the brainstem.[1b,d,2] The vesicular monoamine transporter (VMAT) protein stores serotonin within secretory vesicles at high concentrations (50−270 mM) and low pH (~5).[2d,3] Secretory vesicles transport serotonin from cell bodies to axon terminals along arborized projections that innervate most brain regions, thereby maintaining the vesicle pool size and its stores at steady-state levels for release of vesicular serotonin into the synaptic cleft upon continued neural firing events.[2a,g,4]

Deficient serotonin levels are implicated in the etiology of an array of debilitating neuropsychiatric disorders which include anxiety, bipolar disorder, and clinical depression.[1a,b,2h,5] Selective serotonin reuptake inhibitors (SSRIs) are the most prescribed class of psychotropic medications and utilized as first-line agents to elevate serotonin levels.[1c,4c,6] The persistent administration of SSRIs to serotonergic neurons indirectly dampens negative feedback sensitivity to serotonin release, thereby upregulating the synthesis and transport of serotonin.[4c,6a,7] Accordingly, the enhanced activities promote higher vesicle stores of serotonin and mobilize a larger vesicle pool size in order to accommodate its progressive release.[4a,8]

Molecular imaging tools for monitoring vesicular serotonin levels or the efficacy of SSRIs to modulate the vesicle pool size and its stores primarily include serotonin autofluorescence, fluorescent probes, and radiolabeled ligands.[1a,2b,3b−d,5c,9] Unfortunately, these technologies present certain drawbacks such as displaying limited selectivity, allowing for only indirect observation of serotonin, or requiring concurrent use of invasive biomedical devices. Fluorescent molecular sensors are a compelling technology for achieving the challenging goal of noninvasively imaging serotonin, as they are proven tools that allow for the selective labeling and direct visualization of similar neurotransmitters in neuroimaging applications.[10] For such applications, fluorescent molecular sensors that are able to absorb and emit light within the near-infrared (NIR) optical imaging window (600−1000 nm) are highly desirable because NIR light affords limited background fluorescence and high penetration depths in biological samples.[3c,11]

Herein, we describe the design, synthesis, and evaluation of NeuroSensor 715 (NS715, Scheme 1), which is the first

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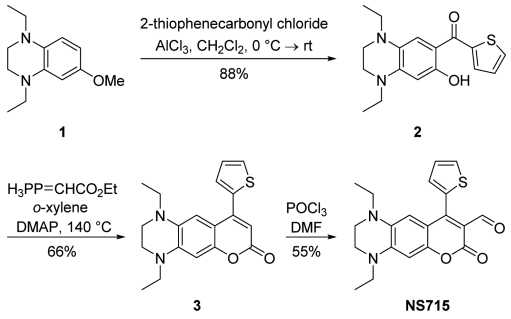
Scheme 1. Synthesis of NeuroSensor 715 (NS715)



molecular sensor to demonstrate a turn-on NIR fluorescence response with selective affinity for serotonin. The sensor is designed to serve as a noninvasive imaging tool that would allow for the selective labeling and direct visualization of electron-rich vesicular monoamine neurotransmitters such as serotonin. A tool for imaging vesicular serotonin would aid efforts toward discerning the spatiotemporal dynamics of its transport and bolster investigations into neuropsychiatric disorders as well as the pharmacological effects of psychotropic medications (e.g., SSRIs) on vesicular serotonin levels.

A short time ago, we developed NeuroSensor 521 (NS521) as a turn-on fluorescent molecular sensor for the monoamine neurotransmitters norepinephrine and dopamine.[10a] NS521 is based on a distinctive platform that consists of an aryl moiety appended to position C4 of a coumarin-3-aldehyde scaffold (fluorophore). The primary amine group of an analyte reversibly forms an imine with the fluorophore aldehyde upon interaction under aqueous conditions. Association with these fluorescence quenching neurotransmitters allows for an enhanced internal charge transfer (ICT) across the activated fluorophore in the excited state, thereby affording a marked turn-on fluorescence response at 521 nm despite the fact that catecholamines are strong fluorescence quenchers. Sensors of this class are designed to capitalize on high concentrations of monoamine neurotransmitters and acidic environment within secretory vesicles of specialized neurons in order to afford selective labeling over typical cellular amines that are present at lower concentrations.[10,12] We validated NS521 as an effective tool for neuroimaging applications by selectively imaging norepinephrine in secretory vesicles of live and fixed cells.

Recently, we established a theoretical model for the rational design of tunable fluorescent molecular sensors based on the coumarin-3-aldehyde scaffold, thereby providing a strategy to identify suitable candidates for imaging neurotransmitters with a simple computational analysis.[10b] The model provides a quantitative basis for predicting the fluorescence response of potential NS521 derivatives toward quenching monoamine neurotransmitters by interrelating the calculated molecular orbital energy values (E_{HOMO} and E_{LUMO}) of both platform components. The model compares, in part, the E_{HOMO} value for a particular pendant aryl substituent to the E_{LUMO} value for the fluorophore in order to predict its fluorescence properties. The fluorescence response of each sensor toward electron-rich neurotransmitters depends on the extent of acceptor-excited photoinduced electron transfer (a-PET) that occurs between its quenching fragment and the NS521 fluorophore (Figure 1). Density functional theory (DFT) calculations provided the calculated E_{HOMO} value for the quenching fragment of norepinephrine, dopamine, and serotonin as well as for the fluorophore of NS521 (Table S1). The E_{HOMO} value for both

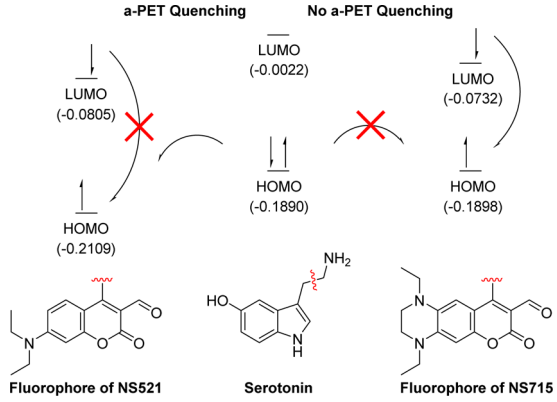


Figure 1. Energy diagram describing design considerations of NS715. Energy values (hartrees) were calculated using DFT.

catechol fragments are comparable to the E_{HOMO} value for the NS521 fluorophore, and therefore the sensor would not undergo a-PET quenching upon careful selection of an appropriate C4-aryl substituent. However, the E_{HOMO} value for the 5-hydroxyindole fragment of serotonin is higher than that of the NS521 fluorophore. As a result, PET quenching could not be overcome, regardless of which substituent we chose.

To develop a molecular sensor that would exhibit a turn-on fluorescence response toward serotonin, we examined many fluorophores that maintained the same structural topology of the coumarin-3-aldehyde scaffold that has been so successful at binding with primary-amine analytes. We settled upon a fluorophore that derives from a 1,2,3,4-tetrahydroquinoxaline (THQ) framework because calculations revealed the resultant fluorophore maintained an extremely high E_{HOMO} value that is nearly identical to the E_{HOMO} value for the 5-hydroxyindole fragment of serotonin (Figure 1), and thereby would avoid PET quenching effects upon association with serotonin. Based on prior work with various pendant aryl substituents, we chose to incorporate a thiophene moiety at the C4 position because it facilitated facile sensor synthesis while maintaining the same energetics of the fluorophore in its C4-unsubstituted form. Calculations of only the fluorophore energetics allowed for strict comparison of the effect that the THQ framework imparts to the coumarin-3-aldehyde scaffold. The calculations also indicated that the THQ-based fluorophore would have a much smaller E_{HOMO}/E_{LUMO} gap value, thereby affording fluorescence emission within the NIR spectral region.

NS715 was prepared as shown in Scheme 1. Compound **1** was acylated and demethylated under Friedel−Crafts conditions to give intermediate **2** in high yield. A high-temperature Wittig reaction with the appropriate phosphorane gave the coumarin core (intermediate **3**) in good yield. Regioselective formylation under Vilsmeier conditions completed the synthesis of NS715.

NS715 was screened with a number of relevant neurotransmitters using both absorption and fluorescence spectroscopy. NS715 produces a large red-shift in its absorption maximum from 500 to 546 nm upon association with serotonin (Figure 2a). Interestingly, the absorption maximum of NS715 is red-shifted by 52 nm compared to NS521 due to the THQ framework. We were delighted to find that exciting NS715 at 559 nm (a common laser line) affords an emission maximum at 674 nm, which is well in the NIR spectral region (Figure 2b). Moreover, we found that the addition of serotonin to NS715

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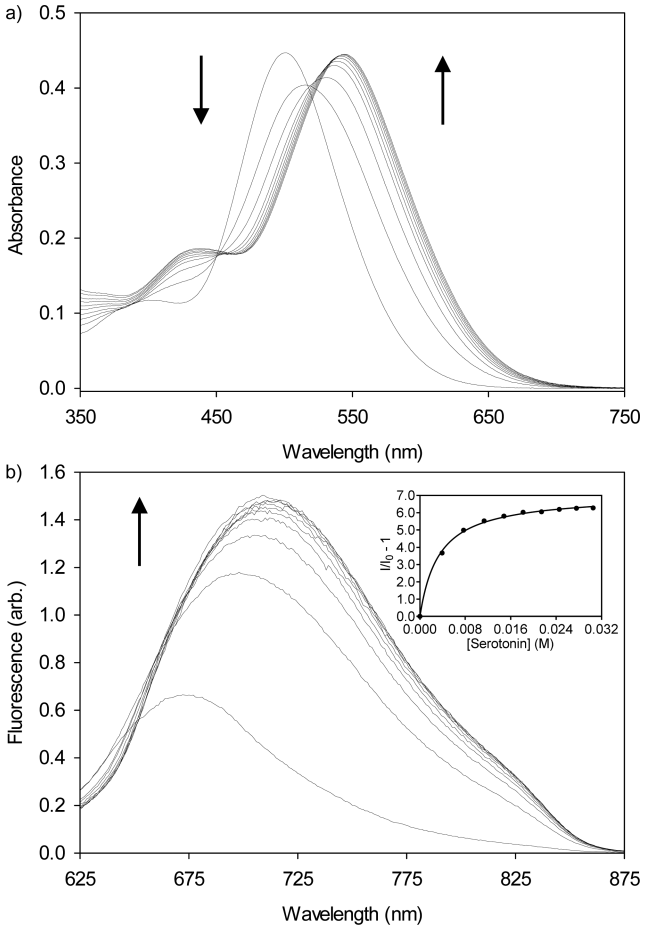

Figure 2. (a) Absorption and (b) fluorescence spectra of NS715 (20 μM) in buffer (50 mM $Na_2S_2O_3$, 120 mM NaCl, pH 5.0, 37 °C) titrated with 0, 20, 40, 60, 80, 100, 120, 140, 160, and 180 μL of a 200 mM solution of serotonin. Titration conditions mimic the environment within secretory vesicles. Inset is the fit to a one-site binding isotherm. I_0 is the initial intensity of the sensor, and I is the measured intensity upon adding serotonin. Intensities were measured at 780 nm. λ_{ex} = 559 nm.

produced a marked increase in fluorescence intensity with a red-shift in its emission maximum to 715 nm. The occurrence of a red-shift in both the absorption and emission maximum upon assocation with a primary amine analyte is similar to that of other fluorescent molecular sensors that are based on the coumarin-3-aldehyde scaffold, whereby imine formation modulates the spectroscopic profile of the sensor by promoting a pronounced push−pull effect and an enhanced ICT across the π-system of the fluorophore.[10a,12,13]

The large red-shifts in the absorption and emission maxima of NS715 permit the sensor to be used in a ratiometric mode, whereby large fluorescence enhancements can be observed by measuring the intensities at longer wavelengths (i.e., 780 nm). Table 1 summarizes the binding and spectroscopic data for the association of NS715 with relevant vesicular neurotransmitters that include the amino acid glutamate and the monoamines norepinephrine, dopamine, and serotonin. NS715 displayed a weak affinity for glutamate along with a modest fluorescence enhancement. NS715 exhibited a much higher affinity for the catecholamines, which is presumably attributed to secondary interactions between the pendant thiophene of the sensor and

Table 1. Association Constants and Spectroscopic Properties for NS715 Binding to Neurotransmitters

neurotransmitter	K_a (M^{-1})[a]	$\Delta\lambda_{abs}$ (nm)[b]	I_{sat}/I_0[c]
serotonin	409	46	8.0
dopamine	145	37	4.0
norepinephrine	129	37	3.4
glutamate	22	30	3.0
epinephrine[d,e]	nd	nd	nd

[a] K_a values were measured using fluorescence spectroscopy. Error in K_a values are ±10% based on triplicate titrations. [b] Bathochromic shift in absorption wavelength using a saturating concentration of neurotransmitter. [c] I_0 is the initial fluorescence intensity of the sensor, and I_{sat} is the maximal fluorescence intensity that was obtained from the theoretical fit to a one-site binding isotherm using a saturating amount of neurotransmitter. Intensities were measured at 780 nm. λ_{ex} = 559 nm. [d] Epinephrine is a secondary-amine analyte which cannot form an imine with NS715, and thereby does not alter its spectroscopic profile. [e] nd = not detectable.

the catechol group. This selectivity and fluorescence response toward the catecholamines is very similar to that of NS521.

Most importantly, NS715 displayed a remarkable 8-fold fluorescence enhancement toward and relatively high affinity for serotonin. Indeed, the affinity of NS715 for serotonin is approximately 19-fold greater than its affinity for glutamate. We presume the selective affinity for serotonin results from the large indole group of serotonin providing stronger secondary interactions with the pendant thiophene of NS715. Clearly, NS715 demonstrates sufficiently high association constants for labeling serotonin within secretory vesicles of specialized cells given its high concentrations (50−270 mM). Further evaluation of NS715 using the secondary-amine neurotransmitter epinephrine resulted in no changes to its absorption and fluorescence properties. Combined with its desirable spectroscopic properties and selective affinity for high concentrations of primary-amine neurotransmitters, NS715 is aptly suited for neuroimaging applications. Thus, we decided to validate the utility of NS715 using chromaffin cells, which serve as a model system for specialized neurons that synthesize, package, and release only a single type of monoamine neurotransmitter such as serotonergic neurons.

Populations of chromaffin cells were separated into distinct norepinephrine-enriched and epinephrine-enriched fractions using standard methods. As designed, NS715 should bind to norepinephrine and strongly fluoresce, but not to epinephrine because it is a secondary-amine neurotransmitter. Both populations of cells were incubated with NS715, washed, and subsequently imaged using confocal microscopy (Figure 3). We utilized the 458 and 633 nm excitation wavelengths to preferentially excite the unbound and bound forms of NS715, respectively. When selectively excited at 633 nm, only the norepinephrine-enriched cells revealed the punctate pattern with strong fluorescence that is expected for labeling the very high concentrations of norepinephrine in secretory vesicles (Figure 3e). The epinephrine-enriched cells showed marginal fluorescence, which can be attributed to NS715 binding to the very low levels of norepinephrine that are present within this type of cell population (Figure 3b). Neither cell population showed any appreciable fluorescence upon exciting at 458 nm, thereby indicating that any potentially unbound sensor was removed during the washing step (Figure 3a and d). Further analysis indicated that the average total cellular fluorescence intensity of the norepinephrine-enriched cells was 15-fold

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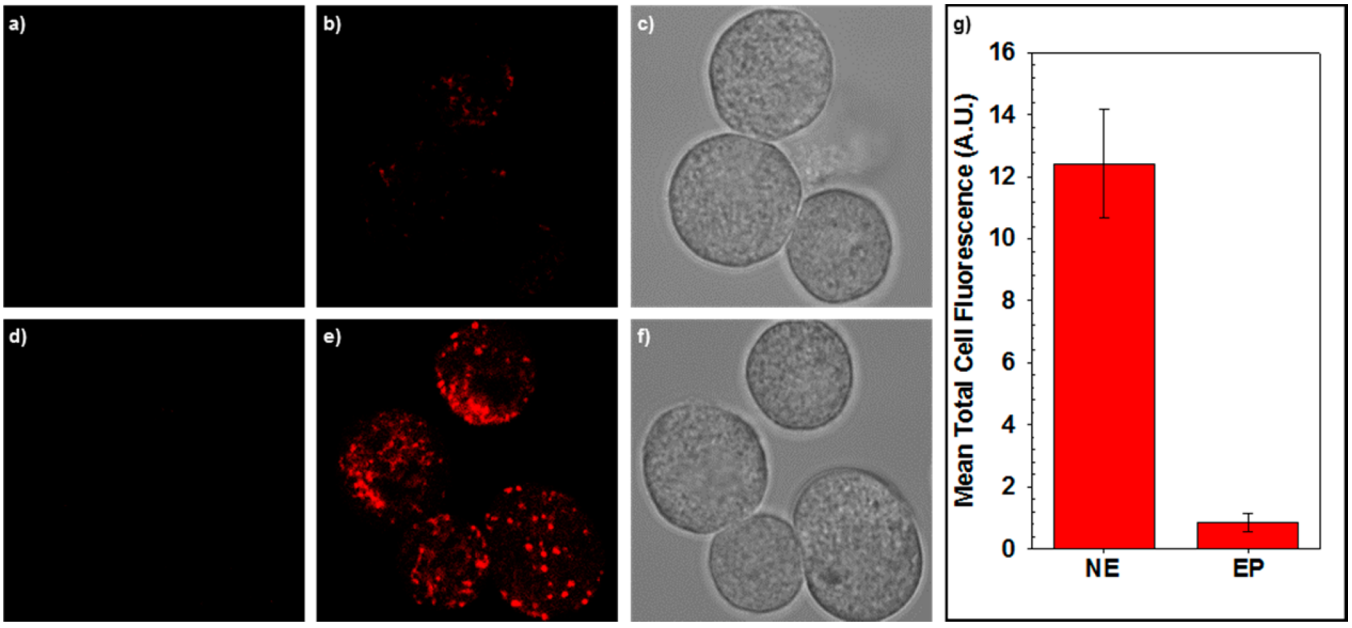

Figure 3. Epinephrine-enriched cells (a–c) incubated with NS715 (10 μM): (a) λ_{ex} = 458 nm; (b) λ_{ex} = 633 nm; (c) brightfield image. Norepinephrine-enriched cells (d–f) incubated with NS715 (10 μM): (d) λ_{ex} = 458 nm; (e) λ_{ex} = 633 nm; (f) brightfield image. Fluorescence was visualized using a 650–710 nm band pass filter. (g) Average fluorescence intensity for norepinephrine- and epinephrine-enriched (NE and EP, respectively) cells was 12.42 ± 1.75 and 0.85 ± 0.29, respectively (n = 12).

higher than that of the epinephrine-enriched cells (Figure 3g). The results revealed that NS715 can differentiate between cell populations that express distinct neurotransmitters.

In summary, we developed NS715 as the first molecular sensor that exhibits a turn-on NIR fluorescence response upon selective detection of serotonin. DFT calculations facilitated the design of a fluorophore based on a coumarin-3-aldehyde scaffold that derives from a THQ framework, which has appropriate energetics for preventing a-PET quenching by serotonin. The utility of NS715 was demonstrated by imaging norepinephrine in secretory vesicles of chromaffin cells, which serve as a common model system for excitatory neurons. NS715 is a noninvasive imaging tool that allows for the selective labeling and direct visualization of select monoamine neurotransmitters such as serotonin.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00235.

UV/vis and fluorescence spectra, synthetic procedures, ^{1}H NMR specctra, ^{13}C NMR spectra, and DFT calculations (PDF)

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Notes

The authors declare no competing financial interest.

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