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Lanthanide complexes as fluorescent indicators for neutral sugars and cancer biomarkers


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Communicated by Josef Michl, University of Colorado, Boulder, CO, May 10, 2006 (received for review February 11, 2006)

Simple water-soluble lanthanum and europium complexes are effective at detecting neutral sugars as well as glycolipids and phospholipids. In solutions at physiologically relevant pH the fluorescent lanthanum complex binds neutral sugars with apparent binding constants comparable to those of arylboronic acids. Interference from commonly occurring anions is minimal. The europium complex detects sialic acid-containing gangliosides at pH 7.0 over an asialoganglioside. This selectivity is attributed, in large part, to the cooperative complexation of the oligosaccharide and sialic acid residues to the metal center, based on analogous prior studies. In MeOH, lysophosphatidic acid (LPA), a biomarker for several pathological conditions including ovarian cancer, is selectively detected by the europium complex. LPA is also detected via a fluorescence increase in human plasma samples. The 2-sn-OH moiety of LPA plays a key role in promoting binding to the metal center. Other molecules found in common brain ganglioside and phospholipid extracts do not interfere in the ganglioside or LPA fluorescence assays.

Results and Discussion

Detection of Neutral Sugars at Physiological pH. A main problem in the detection of neutral sugars with artificial receptors is competitive binding by bulk water. Elevated solution pH is therefore typically required to attain a useful degree of coordination and signal transduction in the most innovative new metal-based detection methods (4, 5). There is an unmet demand for biomimetic sugar-sensing agents that function in neutral buffer solution (4, 5). Because La³⁺ and Ca²⁺ exhibit relatively strong affinity for saccharides as compared with most other metal ions (6, 7), we hypothesize that 1 (Fig. 1) may be useful for detecting sugars in neutral aqueous media. Interestingly, lanthanides can extend their ligand coordination number by the addition of either neutral or charged ligands through ligand-sphere extension, leading to highly coordinated complexes (8).

Addition of saccharides (1.1 × 10⁻³ M) to a solution of 1 (5.53 × 10⁻⁶ M, 0.1 M Hepes buffer, pH 7.0) promotes readily monitored increases in emission (Figs. 1 and 2) (ref. 9 and references therein). Ternary complex formation upon saccharide addition apparently enhances this latter effect. In the ¹H NMR of a solution of 1 and D-glucose in D₂O, the imine protons of 1 exhibit a modest upfield shift, in keeping with analogous salophene–metal complexes upon binding analytes (3) (see supporting information, which is published on the PNAS website).

The continuous variation method indicates a 1:1 stoichiometry among glucose, maltose, maltotriose, and 1 (see supporting information). Glucose, maltose, and maltotriose exhibit binding constants of 500, 1,666, and 2,500 M⁻¹, respectively. These values compare very favorably to those of sugar–boronate complexes (10, 11). This finding is significant because boronic acid-containing fluorophores are currently reagents of choice for sugar detection in aqueous and mixed-aqueous media. The

Nature uses tools such as lectins for the molecular recognition of saccharides. An important mode of lectin binding involves the coordination of a carbohydrate ligand to a metal center. C-type lectins recognize saccharides in a calcium-dependent manner (1). The similar properties of lanthanides and calcium render trivalent lanthanide ions useful substitutes for Ca²⁺ in studying proteins (2). Herein we describe the utility of water-soluble salophene (3)–lanthanide complexes toward addressing three current challenges: (i) the detection of neutral carbohydrates at physiologically relevant pH, (ii) the selective detection of gangliosides, and (iii) the selective detection of lysophosphatidic acid (LPA).
emission enhancements shown herein in the presence of neutral sugars range from \( \approx 25\% \) to \( 60\% \) (Figs. 2 and 3).

Common anions (citrate, phosphate, and pyrophosphate) studied under these conditions promote relatively weaker emission responses (Fig. 3). Additionally, BSA-containing solutions exhibit increased fluorescence only when glucose is present. The concentration of glucose in plasma is typically \( \approx 10^{-3} \). The next most abundant monosaccharides are galactose and fructose, present at concentrations two orders of magnitude less than that of glucose.

The Selective Detection of Gangliosides Under Neutral Conditions. It is well known that an increase or decrease in total sialic acid levels (conjugated plus freely circulating) in biological fluids can indicate the occurrence of certain cancers. The acid-catalyzed liberation of bound sialic acid residues from gangliosides (Fig. 4) for assay typically results in destruction of the analyte (12–16). In the case of enzymatic hydrolysis incomplete sialic acid liberation is a problem (17). Effective sensing agents for sialic acid-containing gangliosides are needed.

Selectivity toward various anionic substrates can be tuned via the appropriate choice of lanthanide metal (18). Importantly, Sillerud et al. (19) have provided precedent for favorable cooperative binding interactions of the oligosaccharide and sialic acid moieties of micellar gangliosides with \( \text{Eu}^{3+} \). For example, the higher affinity of \( \text{Eu}^{3+} \) to GM1 as compared with sialic acid was attributed not only to an electrostatic interaction with the GM1 sialic acid carboxylate but also to secondary interactions with the proximal oligosaccharide hydroxyls, resulting in a coordination shell (Fig. 5).

We thus hypothesize that 2 may afford enhanced signaling in the presence of charged gangliosides as compared with neutral sugars and sialic acid. Compound 2 promotes the detection of sialic acid-containing gangliosides selectively compared with asialo GM1 (Fig. 6). In contrast, 1 affords greater fluorescence enhancement than 2 in the presence of neutral asialo GM1 (see supporting information). The smaller the ionic radius of a lanthanide, the more significant are the intramolecular interactions between its ligands. The salophene ligands of 1 and 2 contain both polar and apolar moieties. The combination of these latter structural features, along with the smaller ionic radius of \( \text{Eu}^{3+} \) compared with \( \text{La}^{3+} \), apparently renders 2 a better substrate for detecting anionic gangliosides compared with 1.

The sialic acid residue of GM1 binds \( \text{Eu}^{3+} \) via multiple coordination sites (Fig. 5). Free sialic acid binding (as predominantly the \( \beta \)-pyranose form) to metals has also been reported (20). The carboxylate, pyranose ring, and glycero side-chain oxygens of sialic acid directly participate in coordination. When sialic acid is titrated with 2 in \( \text{D}_2\text{O} \), the \( ^1\text{H} \) NMR signals corresponding to the protons on the glycerol side chain and pyranose ring undergo substantial peak-broadening. The \( 3\text{H}_{ax} \) proton, on the same side of the pyranose as the carboxylate moiety, is relatively closer to the metal site than \( 3\text{H}_{eq} \). The axial proton resonance of carbon 3 broadens to a greater extent than that of \( 3\text{H}_{eq} \) (see supporting information).

Many compounds are present in typical ganglioside extracts (Figs. 7–9) (21). Major components include free sialic acids, phospholipids, myelins, proline, and glucosamine. These and many structurally related compounds do not interfere with ganglioside detection in neutral buffer solution (Fig. 7). Interestingly, the disialogangliosides (GD1a and GD1b)–2 (Fig. 9)
complexes show stronger emission than the corresponding complex of monosialo GM1-2.

Based on these results we conclude, in agreement with Sillerud et al. (19), that a sialic acid moiety appended to an oligosaccharide sequence leads to enhanced affinity. Comparison of the fluorescence spectra of 2 in the presence of GM1 with neutral asialo GM1 as well as several other analytes suggests that proximal oligosaccharide–sialic acid sequences are important factors leading to signal transduction (Fig. 7).

Gangliosides and neutral sugars can also be monitored by using the well known fluorescent europium(III)–tetracycline (Eu-Tc) complex. The Eu-Tc complex exhibits efficient ligand to metal energy transfer (22). This complex allows for fluorescence monitoring at the common europium emission wavelength of 615 nm rather than at the ligand emission, as in the case of 1 or 2 (Fig. 9). The Eu-Tc complex is well known to exhibit fluorescence emission enhancement upon complexation via displacement of bound water (22). However, the Eu-Tc complex is not as selective as 1 and 2. It exhibits fluorescence emission enhancement in the presence of several neutral sugars and anions (see supporting information).

The Selective Detection of LPA. MeOH solutions containing 2 exhibit increased emission in the presence of commercially available LPA (oleoyl-L-α-LPA Na salt, 5.53 × 10⁻⁶ M λ_ex 360 nm λ_em 400 nm). Solutions containing commercial phosphatidic acid (PA) (3-sn-PA Na salt) exhibit minor fluorescence changes at 400 nm (Figs. 10 and 11), even at millimolar PA levels.
Distinct affinities of LPA and PA for 2 can be interpreted in terms of the magnitude of their corresponding negative charges (23). Intramolecular hydrogen bonding between the phosphate and the 2-sn-OH moieties is observed in the crystal structure of LPA and is known to persist at physiological pH (Fig. 12) (24). The phosphate hydroxyl of LPA is thus more prone to ionization than the phosphate proton of PA. This effect generates a higher negative charge on the LPA phosphate, facilitating proposed binding to 2 dominated by ionic interactions. The free hydroxyl oxygen of LPA may also serve as a cooperative binding site to the lanthanide. It is known that a second coordinating site, especially one containing a hard atom such as oxygen or nitrogen, enhances lanthanide affinity (see also Fig. 5) (25). Indeed, we observe significant broadening of the $^1$H NMR resonances corresponding to protons on carbons 1–3 (Fig. 13) of LPA compared with the other peaks. We propose that this latter feature, in combination with the relatively higher negative charge of LPA compared with PA, should allow selective detection of LPA compared with PA using 2.

Ovarian cancer is a global problem. A main reason for the low survival rate of ovarian cancer is the fact that there is no method for early detection. There is evidence that LPAs (1-acyl-glycerol-3-phosphates), the simplest phospholipids, are promising markers for the early detection of ovarian cancer (26). Current assays for LPA are unsuitable for routine diagnostic and point-of-care use. LPA is relatively difficult to detect in nonpolar lipid extracts. LPA is detected selectively by 2 via an increase in fluorescence in MeOH.

Fig. 14 shows that well known components of phospholipid extracts (27, 28) do not afford fluorescent emission signals comparable to that of LPA in solutions of 2 in MeOH.

We observe a correlation between fluorescence intensity and LPA concentration in MeOH extracts of lyophilized human plasma previously spiked with LPA (as well as LaCl$_3$ to remove neutral interferents; Fig. 15). LPA is detected in the concentration range of $1.83 \times 10^{-7}$ M to $9.15 \times 10^{-5}$ M (Fig. 11). Physiological concentrations of LPA in plasma are approximately $<0.1–6.3$ μM. Danger levels for ovarian cancer are approximately $\leq 43.1$ μM (26).

**Conclusions**

To date, the lack of receptors that effectively mimic lectin binding is largely due to the inability to achieve sugar–metal coordination under neutral conditions. The design of compound 1 is inspired by calcium–saccharide interactions found in C-type lectins. It allows for the successful detection of neutral monosaccharides and oligosaccharides in neutral buffer solution. Our initial studies to date show that complex 2 exhibits enhanced fluorescence emission with anionic lipid analytes that possess proximal hard atom (oxygen) coordination sites, such as the α hydroxyl of LPA and the oligosaccharide hydroxyls of gangliosides. This finding is in excellent accord with prior studies of related systems (25).
Compound 2 can be used to selectively detect (i) sialic acid-containing gangliosides in buffer solution and (ii) LPA in MeOH. These latter results are steps toward developing non-hydrolytic assays for sialic acid and facilitating the detection of LPA, respectively. Our initial focus has been on the selectivity and the signal transduction mechanisms. The complexity of the biomolecules and the nature of the emission (i.e., ligand emission rather than lanthanide emission) render the structural study of the tertiary complexes highly challenging.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich and used without further purification. Gangliosides were purchased from Calbiochem. Phospholipids were purchased from Avanti Polar Lipids. Fluorescence spectra were recorded by using a spectrofluorimeter SPEX Fluorolog-3 equipped with double excitation and emission monochromators and a 400-W Xe lamp. 1H and 13C NMR spectra were acquired on a DPX-250 or DPX-300 spectrometer (Bruker). All δ values were reported in parts per million. Coupling constants are reported in hertz. Fourier-transform IR spectra were acquired on a Tensor 27 IR spectrophotometer (Bruker). MS were acquired on a ProFLEX III MALDI-TOF mass spectrometer (Bruker).

We thank the National Institutes of Health for funding this research through Grant EB 2044 (to R.M.S.). Financial support from the Ministry of Education of the Czech Republic (MSM 6046137307 and LC512) and from the Grant Agency of Czech Republic (203/06/1038 to V.K.) is also gratefully acknowledged.