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Hyperpolarized Xenon-Based Molecular Sensors for Label-Free Detection of analytes

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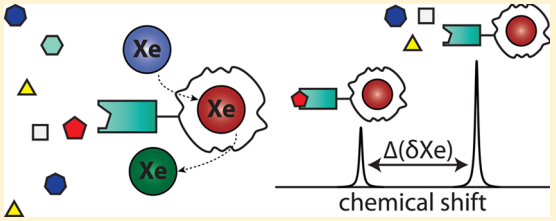
Supporting Information

ABSTRACT: Nuclear magnetic resonance (NMR) can reveal the chemical constituents of a complex mixture without resorting to chemical modification, separation, or other perturbation. Recently, we and others have developed magnetic resonance agents that report on the presence of dilute analytes by proportionately altering the response of a more abundant or easily detected species, a form of amplification. One example of such a sensing medium is xenon gas, which is chemically inert and can be optically hyperpolarized, a process that enhances its NMR signal by up to 5 orders of magnitude. Here, we use a combinatorial synthetic approach to produce xenon magnetic resonance sensors that respond to small molecule analytes. The sensor responds to the ligand by producing a small chemical shift change in the Xe NMR spectrum. We demonstrate this technique for the dye, Rhodamine 6G, for which we have an independent optical assay to verify binding. We thus demonstrate that specific binding of a small molecule can produce a xenon chemical shift change, suggesting a general approach to the production of xenon sensors targeted to small molecule analytes for *in vitro* assays or molecular imaging *in vivo*.



INTRODUCTION

Many analytical methods have been developed for the selective detection of small molecules in trace concentrations within complex mixtures. Applications of these techniques include glucose monitoring[1] in diabetics, the monitoring of organophosphate pesticides in the environment,[2] the evaluation of antibiotic and drug levels in food,[3] and the sensing and recognition of bacteria[4] in aerosols. In general, the most successful of these couple a recognition sensor ("biosensor"), which incorporates a biomimetic binding element targeted to the analyte of interest, with a detection technique that converts the chemical binding response of the sensor to a signal, either optically, electrochemically, or electrically.[5] Critically, many of these approaches do not require covalent modification of the analyte and some can be applied in opaque or impure samples.[5]

Of these methods, those based on a switchable magnetic resonance (MR) response are uniquely promising because they can quantitatively detect biosensor binding in opaque and impure mixtures that cannot be easily interrogated by optical techniques.[6,7] Further, MR sensors can strongly amplify a binding response by proportionally affecting the signal of a much more abundant species, such as the solvent, in response to the recognition of a dilute analyte. In the most successful of these MR biosensors,[8] contrast is generated by the aggregation of functionalized paramagnetic nanoparticles in the presence of the analyte, resulting in a large change in the NMR signal of the abundant water solvent. While successful in many cases, aggregation-based MR biosensors necessarily couple the dynamics of analyte detection with those of signal transduction, making it difficult to independently optimize both.

To develop a sensing system that preserves the beneficial characteristics of MR detection while overcoming some of these difficulties, here we report the use of xenon-based molecular sensors in the detection of small molecules in aqueous solution. Instead of the solvent itself, our sensors utilize dissolved xenon gas as an inert reporting medium for a MR assay. Xenon is an ideal medium on which such a sensor can operate: it is not naturally present in most samples; it is soluble in both hydrophobic and hydrophilic solvents;[9] and its nuclear spin can be hyperpolarized through spin exchange optical pumping,[10] generating NMR signals of strength comparable to those of water in conventional experiments, even when xenon is present in micromolar concentrations. Furthermore, xenon spectra span a very large range of chemical shifts (>200 ppm), because the high polarizability of the xenon atom renders its NMR spectra extremely sensitive to the local physiochemical chemical environment.[11−16]

In our system, signal transduction is based on a change in the NMR properties of the sensor upon its noncovalent association with the analyte. Specifically, our sensors are based on a small organic cage molecule, cryptophane-A, with which xenon

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transiently associates in solution, resulting in a large change in chemical shift (>100 ppm).[17] When such a sensor is targeted and bound to an analyte of interest, the electronic environment of the encapsulated xenon is further perturbed, resulting in a modest change (<10 ppm) in its chemical shift.[18] The high resolution of xenon NMR facilitates the selective spectroscopic manipulation of xenon populations corresponding to free xenon, xenon bound in the cage, and xenon bound in a cage which, itself, has bound the analyte. Such sensors have been used to report strong binding events, such as between a biotinylated sensor and avidin[17,19] and DNA hybridization.[20] However, they have not previously been employed as sensors of weak binding to small molecule analytes.

In this study, we wanted to demonstrate that specific binding of a small molecule can produce a xenon chemical shift change. Toward this, we explored the ability of xenon molecular sensors to detect analytes to which they are only weakly associated. To do so, we coupled the cage to a peptide recognition moiety whose sequence could be varied combinatorially. This approach, using peptides or peptoids as the variable element, has also been used for the design of unrelated MRI contrast agents.[21−23] It is successful because peptides are easily synthesized in a combinatorial manner and can be readily attached to cryptophanes[17,24] Using colorimetric methods, we screened this sensor library[25−27] for weak binding to a common organic dye, Rhodamine 6G, and then explored the correlation of the xenon NMR response to the optically detected binding. Though optical dyes are frequently used as labels for analyte detection, in our NMR experiments the dye molecule itself was detected in a label-free fashion, indicating the potential to detect other analytes with the same method.

EXPERIMENTAL SECTION

Design of Peptide Library. For our library of peptide receptors, we designed a 12 amino acid sequence with four variable positions, H_2N-KX_4X_3PGX_2X_1GWKKG-CO_2H (Figure 1), that included a D-Pro-Gly unit between the four randomized positions to encourage formation of secondary structure in the peptide moiety.[28,29] All 20 natural amino acids were used in each of the four variable positions (indicated by the **X** positions), corresponding to 160 000 total members. The library was synthesized on Tentagel S poly(ethylene) grafted poly(styrene) resin beads using standard Fmoc solid-phase peptide chemistry and a split-and-pool technique.[25,26] The five amino acid sequence GWKKG was appended to the variable regions to provide the library members enough mass to distinguish their signals from matrix noise peaks during MALDI-TOF MS analysis (Trp also provided a fluorescence detection handle). To facilitate sequencing by mass spectrometry, a truncation ladder was incorporated by capping 10% of the peptides after the coupling of each variable position.[30,31] The sequence on each bead was determined after cleaving by the unique isotope pattern of the truncated species using the mass difference between molecular ions (see Figure S2 for representative analysis of peptide sequence using MALDI). In initial experiments, the N-terminus of the peptide library was capped with pyrene as a surrogate for the hydrophobic cryptophane cage. After the initial screening and optimization experiments, the cage was introduced at the N-terminal position using NHS ester coupling chemistry (see Supporting Information for more details).

Screening for Selective Binding Sequences. We elected to use commercially available visible dyes for the initial screening of the library, making binding sequences readily identifiable. The sensor library was incubated with a variety of dyes, and colorimetric screening conditions were optimized to find a dye with specific binding to a small number of library members (<2%) based on visual inspection. Upon incubation of the bead library with a 10 μM solution of Rhodamine 6G dye for 1 h, followed by washing to remove unbound

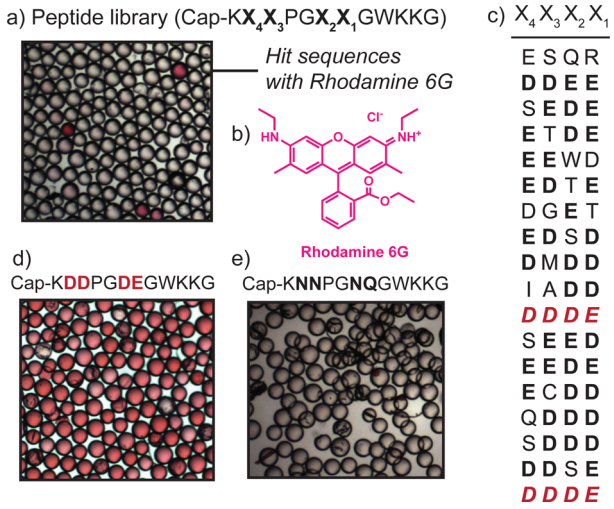

Figure 1. Design, synthesis and screening of a 160 000 member peptide library of sequence KX_4X_3PGX_2X_1GWKKG. (a) Incubation of the peptide library (pyrene capped) with 10 mM Rhodamine 6G dye (stringent conditions) gave very few hits per screen. (b) Structure of the model analyte Rhodamine 6G, used to confirm peptide-analyte binding both by a colorimetric assay and by xenon chemical shift change. (c) Sequencing was performed by cleaving the peptide species from selected "hits" and identifying the ladder peptides using MALDI-TOF. A 'hit' sequence (D-peptide, red) and a control peptide were resynthesized and capped with pyrene/cage. (d) Visual confirmation of binding interaction of "hit" D-peptide cage with Rhodamine 6G incubation. (e) Control peptide (N-peptide cage) incubation with Rhodamine 6G under same conditions shown no color change.

dye, we observed a small number of peptide sequences that showed binding (Figure 1a,b). Separating the beads on a watch glass allowed the visual detection and manual extraction of dark-pink beads for sequencing. The library was screened multiple times using this procedure, and the peptide sequences that were identified to bind Rhodamine 6G are tabulated in Figure 1c. These sequences showed a consensus for aspartate and glutamate residues in all four variable positions. The identified motif (X_4, X_3, X_2, X_1 = D, D, D, E) was selected for further verification experiments and validation.

RESULTS AND DISCUSSION

Colorimetric Confirmation of Binding Specificity. For other studies of dye binding, the consensus motif with the sequence H_2N-KDDPGDEGWKKG-CO_2H (D-peptide) was resynthesized. We chose as a control peptide the sequence H_2N-KNNPGNQGWKKG-CO_2H (N-peptide), which has neutral, isosteric asparagine and glutamine residues in place of their charged counterparts. Following completion of the colorimetric screening experiments involving pyrene-capped peptides, the reaction conditions for capping the N-termini with the cryptophane cage were optimized by varying the time and reagent concentrations (see Supporting Information for synthetic details and figures).

Upon subjecting two sets of beads, each bearing one of the peptides, to identical screening conditions, it was visually confirmed through distinct color contrast that the D-peptide-pyrene construct bound the dye more strongly than the N-peptide-pyrene construct. These experiments produced the same results when cryptophane cage was at the N-terminus of the peptide, confirming that the binding interaction was between the dye and the D-peptide sequence, not the pyrene (Figure 1d,e). The specificity of this sequence for Rhodamine 6G dye was confirmed by incubating both D- and N-peptide-

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pyrene with several other similar dyes, including Rhodamine B, TAMRA, Coomassie Brilliant Blue, and Rhodamine B piperazine amine.[32] No other dye bound specifically to the D-peptide under the same incubation and wash conditions. Representative images of these trials with other dyes are shown in Figure S7.

^{1}H NMR Confirmation of Binding Specificity. Having identified a peptide that bound Rhodamine 6G and a control sequence that did not, we verified the interaction between the D-peptide-cage and the dye by using nuclear Overhauser effect spectroscopy (NOESY) to measure through-space interactions between the protons. NOESY spectra were acquired for five samples: 250 μM dye only, 200 μM D-peptide-cage with and without 250 μM dye, and 200 μM N-peptide-cage with and without dye. Three samples, the free dye, the D-peptide-cage without the dye, and N-peptide-cage without the dye, produced spectra containing cross peaks corresponding to intramolecular interactions within the peptide-cage construct or within the dye molecule. The spectra of both the D- and N-peptides in the presence of dye revealed cross peaks in the region corresponding to dye-peptide interactions. However, there were many more cross peaks in the D-peptide-cage sample (containing dye), and they were more intense than those in the N-peptide-cage sample containing dye. This observation indicated a stronger interaction between the dye and the D-peptide-cage than with the N-peptide-cage.

Furthermore, there were peaks in the D-peptide-cage sample containing dye that corresponded to through space interactions to other dye protons, but these cannot be intramolecular interactions given the dimensions of a single dye molecule. It is possible that these cross peaks resulted from exchange transferred NOEs of dye molecules through the peptide or that multiple dye and peptide subunits associate, leading to the physical proximity of dye molecules. While the NOE data were not sufficient to define the nature of the peptide-dye interactions completely, they verified that there was a difference in the interactions of the dye with the D- and N-peptides, confirming our visual evidence that the library produced a receptor for the Rhodamine 6G analyte.

Xenon-Based NMR Detection of the Analyte. Having verified that Rhodamine 6G interacts differently with D-peptide-cage than with N-peptide-cage, both colorimetrically and with proton NMR, we used xenon NMR to detect the binding of the dye through chemical shift changes. Five samples of each peptide-cage construct were prepared, each having the same amount of peptide-cage and concentrations of dye from 0 to 1 mM in 0.25 mM steps. Each sample was loaded into a NMR tube that was connected to a xenon hyperpolarizer.[33,34] Following delivery of hyperpolarized xenon to the solution, the xenon spectrum was acquired using a 90° radiofrequency pulse. Three peaks were apparent in each spectrum, as shown in Figure 2a: a Xe_{gas} peak, which is used as a chemical shift reference set to 0 ppm, a peak corresponding to xenon encapsulated in the cage (Xe@cage) near 60 ppm, and a Xe_{aq} peak near 190 ppm. At higher dye concentrations, both the Xe@cage and the Xe_{aq} resonance frequencies shifted: the Xe@cage resonance frequencies shifted downfield, while the Xe_{aq} resonances shifted upfield. This was observed for both the D- and the N-peptide-cage samples. The frequency of the Xe@cage peak, which is the signal most sensitive to environmental perturbations, was affected much more strongly in the D-peptide-cage samples than in the N-peptide-cage samples. This indicated that xenon could indeed detect the complexation of the dye to the D-peptide-cage without the use of external labels. The changes in chemical shift are shown in Figure 2b.

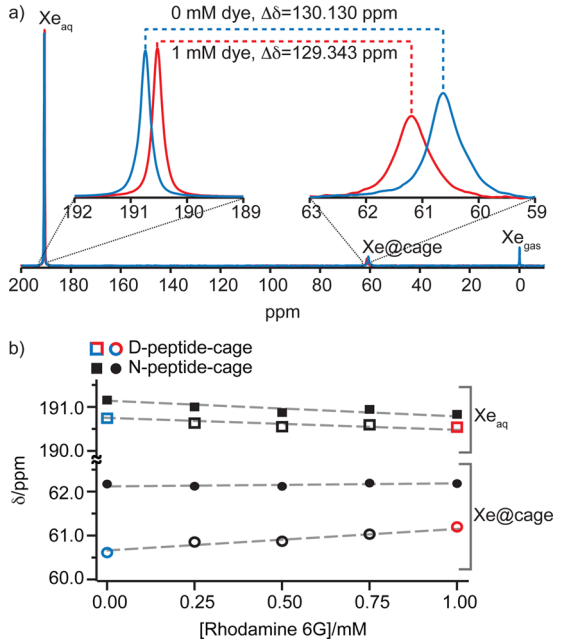

Figure 2. Xenon NMR spectra of two D-peptide-cage samples. (a) ^{129}Xe NMR spectra were collected for 200 μM D-peptide-cage samples containing 0 mM Rhodamine 6G dye (blue spectrum) and 1 mM dye (red spectrum). Each spectrum is referenced to the Xe_{gas} peak at 0 ppm; the other peaks correspond to Xe@cage (~60 ppm) and Xe_{aq} (~190 ppm). Both the Xe@cage peak and Xe_{aq} peak shift upon addition of dye. (b) The chemical shift positions are plotted for the D-peptide-cage (hollow symbols) and N-peptide-cage (filled symbols) samples at various dye concentrations between 0 and 1 mM. The red and blue symbols correspond to the spectra shown in (a). The difference between the Xe_{aq} and Xe@cage signals changes more in the D-peptide-cage sample than in the N-peptide-cage samples upon addition of dye, indicating a stronger interaction between the dye and the D-peptide-cage sensor molecule. All chemical shift positions are determined with Lorentzian fit parameters to the NMR spectral data. Trend lines in (b) are added as a guide to the eye.

Since the changes in Rhodamine 6G concentration caused shifts for both the Xe_{aq} and Xe@cage NMR signals, the frequency difference between those peaks, $\Delta(\delta Xe_{aq} - \delta Xe@cage)$, is the property best suited for comparison of D-peptide-cage and the control N-peptide-cage samples. The results are listed in Table 1. While both the D- and the N-peptide-cage samples showed a decrease in $\Delta(\delta Xe_{aq} - \delta Xe@cage)$ upon dye addition (increasing the dye concentration from 0 to 1 mM),

Table 1. Differences between Chemical Shift Positions of the Xe_{aq} (δXe_{aq}) and Xe@cage ($\delta Xe@cage$) Peaks for Both the D- and N-Peptide-Cage Samples Containing Varying Rhodamine 6G Dye Concentrations

[Rho 6G]	D-peptide-cage	N-peptide-cage
mM	$\Delta(\delta Xe_{aq} - \delta Xe@cage)$/ppm	
0.00	130.12	128.98
0.25	129.78	128.88
0.50	129.68	128.75
0.75	129.56	128.75
1.00	129.34	128.65

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the decrease was much more pronounced for the D-peptide cage than the N-peptide cage.

This result establishes an observable spectroscopic change due to the interaction of Rhodamine 6G dye with D-peptide-cage in solution, validating the use of xenon-based molecular sensors in detecting analytes. In future experiments, binding characteristics will be studied more thoroughly. Also, stronger and more specific binding between the analyte and the sensor could likely enhance this spectroscopic change, perhaps by using a longer or more structured peptide in the sensor molecule. Finally, the principal limitation of this example is the small chemical shift change upon binding. An approach in which the primary combinatorial library is screened by MRI rather than optical methods may reveal peptide sequences that produce a greater chemical shift change upon binding their targets. Approaches that exploit paramagnetism, in analogy to successful ^{1}H PARACEST experiments, may also be fruitful.

CONCLUSIONS

The aim of this paper is to demonstrate that specific binding of a small molecule can produce a xenon chemical shift change. We have demonstrated that xenon-based molecular sensors are capable of detecting weak binding to analytes of interest in solution, without covalent modification of the analyte. Unlike conventional MR approaches, ours uses a hyperpolarized sensing medium for high sensitivity, decouples the MR response from the mechanism of analyte recognition, and, in principle, is compatible with operation in a mixed-phase device such as a microfluidic chip.

In combination with remotely detected microfluidic NMR,[35] this may eventually permit multiple analytical assays to be conducted in parallel and detected with a single detector. Though we have used short peptides as a recognition moiety, longer peptides, peptoids,[36,37] or DNA aptamers may be used as the variable end of the sensor to increase affinity for some targets.[38] Finally, the same methods described here may be used to produce molecular imaging agents for studies of relevant targets *in vivo*.

ASSOCIATED CONTENT

Supporting Information

Synthetic details, data on the evaluation of binding, and proton NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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