Anal. Chem. **2005,** *77,* 4008−4012

NMR-Based Biosensing with Optimized Delivery of Polarized ^{129}Xe to Solutions

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Laser-enhanced (LE) ^{129}Xe nuclear magnetic resonance (NMR) is an exceptional tool for sensing extremely small physical and chemical changes; however, the difficult mechanics of bringing polarized xenon and samples of interest together have limited applications, particularly to biological molecules. Here we present a method for accomplishing solution ^{129}Xe biosensing based on flow (*bubbling*) of LE ^{129}Xe gas through a solution in situ in the NMR probe, with pauses for data acquisition. This overcomes fundamental limitations of conventional solution-state LE ^{129}Xe NMR, e.g., the difficulty in transferring hydrophobic xenon into aqueous environments, and the need to handle the sample to refresh LE ^{129}Xe after an observation pulse depletes polarization. With this new method, we gained a factor of >100 in sensitivity due to improved xenon transfer to the solution and the ability to signal average by renewing the polarized xenon. Polarized xenon in biosensors was detected at very low concentrations, ≤250 nanomolar, while retaining all the usual information from NMR. This approach can be used to simultaneously detect multiple sensors with different chemical shifts and is also capable of detecting signals from opaque, heterogeneous samples, which is a unique advantage over optical methods. This general approach is adaptable for sensing minute quantities of xenon in heterogeneous in vitro samples, in miniaturized devices and should be applicable to certain in-vivo environments.

The noble gas ^{129}Xe has gained considerable interest as a zero-background, sensitive probe for NMR to report on changes of pore size, pore shape, and the chemistry of specific sites, e.g., solid nanoporous materials[1−3] and proteins in solution.[4−11] Even extremely small changes in pressure, concentration, molecular conformations, or isotopes in the local sample environment can be detected by ^{129}Xe nuclear magnetic resonance (NMR) spectroscopy.[5,9,12,13] The versatility of using Xe in applications arises from two features: the extremely large chemical shift range of xenon, giving a rich spectroscopic fingerprint;[10,14,15] and the possibility of laser optical pumping of xenon, which enhances Xe polarization and therefore the NMR signal amplitude by 4−5 orders of magnitude.[16−21] The focus of the work described here is to increase sensitivity in applications of laser-enhanced (LE) ^{129}Xe as a biosensor in aqueous solutions.

Conventional solution-state ^{129}Xe NMR utilizing dissolved LE ^{129}Xe has been performed in batch mode.[4−11,22−27] This requires collection of a batch of LE ^{129}Xe from the polarizer by freezing it out of the buffer gases and thawing it into an evacuated, gastight glass tube that contains the sample. The sample is previously deoxygenated by freeze−pump−thawing to prevent relaxation of LE ^{129}Xe by oxygen's unpaired electrons. The sample tube is then shaken vigorously to dissolve LE ^{129}Xe and immediately inserted into the magnet for NMR measurement. The sample can be

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10.1021/ac0500479 CCC: $30.25
Published on Web 05/18/2005

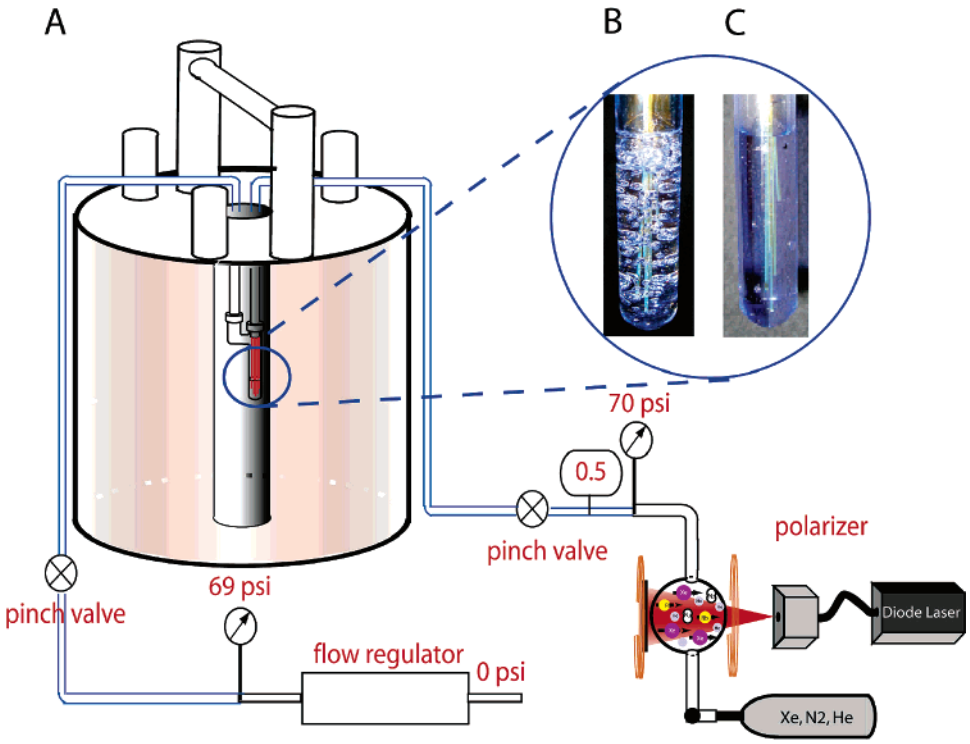

Figure 1. (A) Bubbling setup. Laser-enhanced ^{129}Xe gas (1%) together with a mixture of N_2 (10%) and He (89%) is produced in a commercial polarizer (Amersham Health) at 70 psig and directly routed into the NMR sample tube via a bubble dispenser inside the NMR probe and magnet. The inlet and outlet of the sample tube can be opened and closed in a programmable way for flow control. Here, pneumatically actuated pinch valves were used to stop the flow for the inlet and outlet by pinching the outside of the tubing. Pinch valves were used instead of solenoid-actuated valves in order to minimize depolarization by contact of HP ^{129}Xe with metal components and magnetic fields produced by a solenoid-actuated valve. Typically, bubbling was continued for 30−60 s, at a flow rate of 0.5 SLM and stopped for 10 s to allow the sample to equilibrate before the signal was acquired. (B) The perfusion of solution with continuous gas bubbles was realized through a bundle of five fused-silica capillaries that extend into the solution. (C) Continuous bubbling was stopped only during the acquisition of NMR signal to minimize line broadening due to heterogeneous magnetic susceptibilities in a bubbling sample. Opening and closing of the valves was programmed into the pulse sequence.

repeatedly removed, shaken, and reinserted for detection until the polarization reservoir of slowly relaxing gaseous LE ^{129}Xe above the liquid sample is depleted. The limitations of this approach are not only that it is time-consuming and tedious but also that it may cause damage to sensitive biological samples such as proteins and membranes, which may not be amenable to degassing and shaking. Another limitation is that the number of possible repetitions for a single batch of xenon is restricted by the size of the gas reservoir.

Here we present a new method for the more effective utilization of LE ^{129}Xe for NMR biosensor applications that overcomes the shortcomings of batch-mode LE ^{129}Xe NMR. Polarized ^{129}Xe gas is delivered into solution almost continuously (*bubble* mode), stopping only for NMR acquisition. This procedure directly enhances the signal, and makes extensive signal averaging practical.

EXPERIMENTAL SECTION

The setup for bubble-mode LE ^{129}Xe NMR consists of a pressurized apparatus (~70 psig) that routes LE ^{129}Xe gas directly from the xenon polarizer through the sample tube located inside the NMR probe and magnet, as diagrammed in Figure 1A. The sample tube (o.d. = 10 mm) is modified with a gastight inlet containing five capillary tubes, which extend into the sample solution for perfusion, and with a gastight outlet elbow. For a bubbling setup using a 10-mm-diameter NMR tube, we found that a bundle of equally spaced capillaries works better than a glass frit with smaller pores. The problem with using a frit was that the size of the commercially available frit leaves only a small spacing between the frit and the inner wall of the glass tube so that the escaping bubbles fuse with each other or with the glass wall causing the sample to bubble out of the NMR tube. The LE ^{129}Xe gas can flow through the sample in a controlled manner by maintaining a small pressure gradient (1−2 psi) across the sample tube by means of a flow regulator at the system outlet. Such regulated bubbling can be viewed as perfusion in which the contact surface and time of the small gaseous bubbles with the solution is maximized, allowing for efficient dissolution of xenon. The bubbling (Figure 1B) is stopped (Figure 1C) during NMR signal acquisition by closing both valves on each side of the sample. This provides sample homogeneity and also minimizes magnetic field gradients caused by susceptibility mismatches at bubble interfaces. The control of both valves is programmed into the pulse sequence and triggered by the NMR spectrometer.

A further dramatic sensitivity enhancement of the biosensor signal was achieved by exploiting the exchange of the xenon in and out of the biosensor cage. In the literature, xenon exchange has been utilized in various circumstances for signal enhancement.[11,28] Signal-to-noise ratio (SNR) can be gained by selectively

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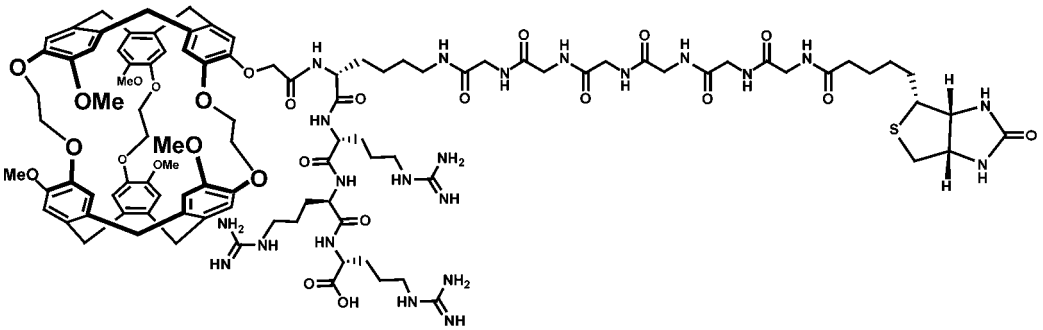

Figure 2. Structure of the water-soluble biotinylated xenon biosensor consisting of a cryptophane-A cage that has been attached via a peptide bond to a branched peptide K((G)$_6$-biotin)RRR.

observing only the encapsulated ^{129}Xe and repeating after delays that allow for exchange of the cage-bound xenon with fresh polarized xenon from the solution.[11] We refer to this method as exchange signal averaging and incorporate it into the bubble-mode pulse sequence, which allows for multiple (>500) observation pulses within each stopped bubble cycle. An E-BURP rf pulse with narrow bandwidth was used to selectively excite the encapsulated xenon resonance. E-BURP pulses are very sensitive to even small mis-settings of the pulse length,[29] which is why xenon gas and xenon in solution were also detected but with greatly attenuated sensitivity as can be seen in the spectra.

RESULTS AND DISCUSSION

The performance of this bubble-mode LE ^{129}Xe NMR technique was demonstrated using a water-soluble xenon biosensor that detects binding of biotin to avidin. The biosensor is composed of a ^{129}Xe-encapsulating cryptophane-A cage that has been "functionalized"[6] by attaching a biotinylated branched peptide (Figure 2). Cryptophane-A carboxylic acid was synthesized as previously described[11] and attached to the deprotected N-terminus of a small hydrophilic peptide [(ϵ-G$_6$-biotinyl)K-R$_3$COOH] that was synthesized by conventional solid-phase methods (Fmoc chemistry; HBTU/HOBT/DIEA acylation; deprotection in TFA with thioanisole; purity of crude product was >95% by FTICR-MS; calculated mass 2102.957 Da; found mass 2102.957 Da). As has been previously described, this synthetic approach produces an equal mixture of two biosensor diastereomers, which leads to two unique xenon biosensor resonances.[11] Figure 3A shows a complete spectrum of ^{129}Xe in an aqueous solution of 3 μM concentration of this xenon biosensor. The most upfield peak corresponds to xenon gas originating from residual bubbles (or gas in the capillaries) and appears broad because bubbles create local field inhomogeneities. The gas peak is used for spectral referencing at 0 ppm. ^{129}Xe is in slow exchange (on the NMR chemical shift time scale) between the biosensor and bulk solution, yielding a pair of distinct resonances for ^{129}Xe encapsulated in the biosensor diastereomers with chemical shifts of ~65 ppm and a single resonance at ~190 ppm for ^{129}Xe dissolved in solution. A diagram illustrating the xenon biosensor in solution is presented in Figure 3B together with expansions of the xenon biosensor peaks at 65.3 and 64.2 ppm.

^{129}Xe NMR measurements in bubble mode, as opposed to batch mode, resulted in a far higher and more reproducible SNR for both the ^{129}Xe in solution and the ^{129}Xe biosensor. The SNR gains can be attributed to a higher concentration of dissolved LE ^{129}Xe, which is due to more efficient perfusion of the solution with gas, and/or higher LE ^{129}Xe polarization. A higher polarization of ^{129}Xe can be accounted for by more efficient preservation of the polarization because it does not undergo the phase transitions during preparation and delivery into the sample. LE ^{129}Xe also travels through reproducible magnetic field gradients during transport compared to batch mode where the sample tube is manually removed, shaken, and reinserted into the magnet.

In the batch mode, typically 300 μM or higher concentrations of biosensor were examined in order to obtain reasonable SNR for the ^{129}Xe biosensor signal. However, in bubble mode, biosensor concentrations as low as 1.5 μM for each biosensor diastereomer were detected with a SNR of ~5 (Figure 3B). Because the xenon occupancy in the biosensor is ~60% under these conditions and the xenon in the gas mixture[30] is only 27% ^{129}Xe (natural abundance), each peak in the spectrum in Figure 3B represents a concentration of ~250 nM LE ^{129}Xe. This was calculated using a xenon binding constant[31] estimated at 6000 M^{-1} and xenon in solution estimated at ~0.25 mM by assuming a solubility of 4.4 mM/atm Xe. For this sample, 99.2% of the xenon was dissolved in solution while 0.8% was encapsulated in the cage. Additional optimization of the exchange signal averaging pulse sequence by matching the timing and the pulse flip angle of the sequence for each biosensor system specifically may further improve the sensitivity to the tens of nanomolar range. In addition to the inherent sensitivity advantage in bubble mode, the bubbling approach allows much shorter sample preparation times since the sample does not have to be deoxygenated, and the LE ^{129}Xe does not have to be collected by freezing out. Microcoil NMR detection has been shown to be effective at detecting very small masses of samples in nanoliter volumes,[32,33] but this corresponds to concentrations of many millimolar. Such high concentrations often cannot be reached for biomolecules, particularly large proteins due to their modest solubility, limiting the application of microcoils for their detection.

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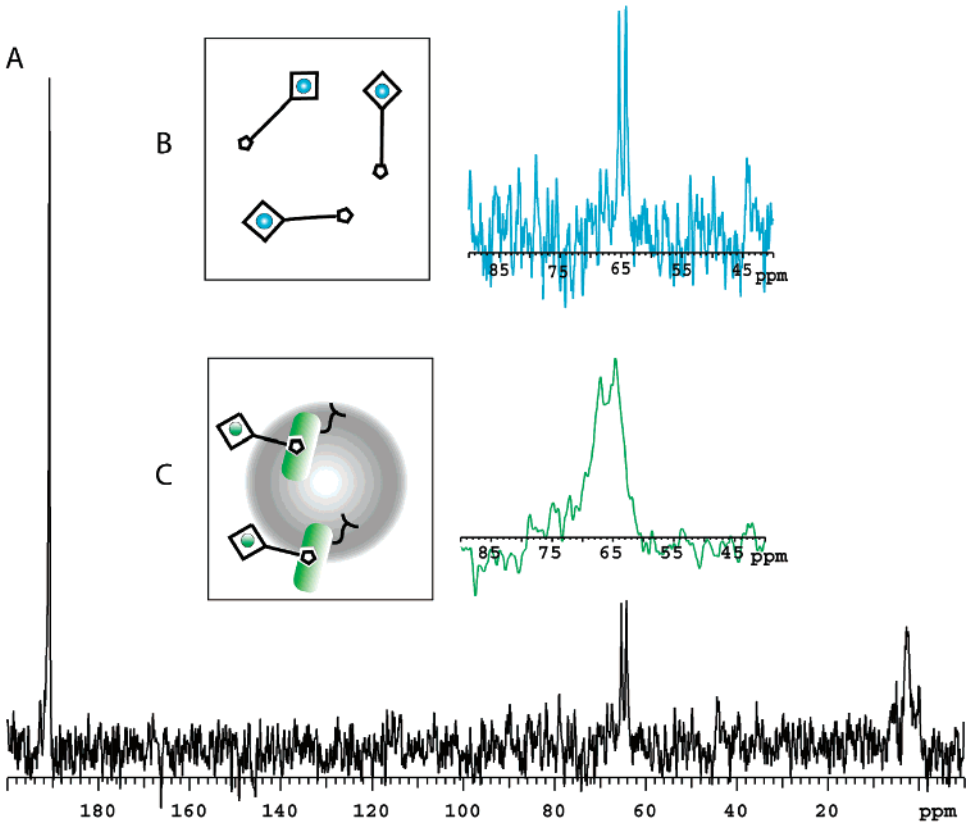

Figure 3. (A) The ^{129}Xe spectrum of 3 μM biosensor in 1.5 mL of 50% D_2O for locking purposes. The spectrum is referenced to xenon gas at 0 ppm. Stopped-flow times of 30 s flow and 10 s stopped were used for the acquisition of 4 scans, each consisting of 512 exchange signal averaging loops with an exchange delay of 60 ms for acquisition. (B) An expansion of (A) around the diastereomeric resonances at ~65 ppm of 3 μM biosensor shows that they are well resolved with each corresponding to 1.5 μM diastereomer concentration. (C) An expansion of the same region of the spectrum for bead-immobilized biosensor in a 50% slurry (by volume) of immobilized-avidin acrylamide beads (Ultralink Immobilized Neutravid Plus, Pierce) that have a maximum biotin binding capacity of 80 μM in a 1.5-mL 50% D_2O solution. The cartoons illustrate the xenon encapsulating biosensor (B) and the bound biosensor to the avidin-immobilized bead (C). The spectrum is referenced to xenon gas at 0 ppm. The solution peak region was different than that for water, as shown in Figure 5. Stopped-flow times of 30 s flow and 9 s stopped were used for the acquisition of 32 scans, each consisting of 50 exchange signal averaging loops with an exchange delay of 60 ms for acquisition. The number of exchange signal averaging loops was optimized for each sample to maximize overall SNR. For a given exchange delay, the optimum number of exchange loops depends on biosensor concentration and xenon exchange rate for the biosensor in solution.

Bubble-mode LE ^{129}Xe NMR can be applied to the detection of very dilute analytes in complex and heterogeneous environments. We demonstrate, as an example, biotin−avidin binding taking place on agarose (45−165-μm diameter) and acrylamide beads (50−80-μm diameter), dispersed in water, in which the avidin is covalently anchored to the bead. The utilization of beads can be a simple means to introduce and recover an immobilized sensor from aqueous samples. Bead-immobilized biosensor was prepared by adding excess biotinylated biosensor (Figure 2) to a 50% (v/v) slurry of 6% cross-linked avidin-functionalized acrylamide (Ultralink Immobilized Neutravidin Plus, Pierce Biotechnology) or agarose beads (Immobilized Avidin, Pierce Biotechnology). Excess biosensor was removed by washing the beads multiple times with 0.1% sodium dodecyl sulfate. The ^{129}Xe spectrum of a 50% slurry of biosensor prepared beads in water shows an upfield peak at 0 ppm from residual xenon gas bubbles, a peak at ~65 ppm from bead-immobilized biosensor, and resonances at ~193 ppm from dissolved ^{129}Xe.[34] The sensitivity of the encapsulated ^{129}Xe chemical shift to the binding of avidin to the biotinylated linker has been previously demonstrated.[6] Figure 3C shows the region around the biosensor signal for biosensor immobilized on acrylamide beads, with an estimated sensor concentration of 80 μM. No biosensor signal was observed from control samples, made by saturating the avidin with excess biotin before adding excess biosensor and washing, confirming that the resonance in Figure 3C arises from biosensor specifically bound to bead-immobilized avidin. Figure 4 shows the region around the solution resonances for both agarose and acrylamide bead slurries. The cartoon in Figure 4A illustrates the dissolved ^{129}Xe free in solution and associated with the bead matrix. Parts B and C in Figure 4 show a closeup of this region for separate samples of agarose and acrylamide beads where the sharper peak corresponds to ^{129}Xe in solution and the broader peak to ^{129}Xe associated with the bead matrixes. These spectra show how the ^{129}Xe chemical shift is indicative of the moderately different bead environments. Studies are currently underway to determine the source of this sensitivity, which may affect the system's exchange properties,

(34) The 50% slurries of both agarose and acrylamide beads will begin to settle during the (10-s) stopped-flow time prior to signal acquisition and distort magnetic field homogeneity, which leads to significantly broadened lines compared to those from the biosensor dissolved in a homogeneous solution. However, the signal line width obtained by NMR signal acquisition for shimming during the course of continuous bubbling at a reduced flow rate can accurately reproduce the (in-)homogeneity of the sample at the time of stopped-flow acquisition.

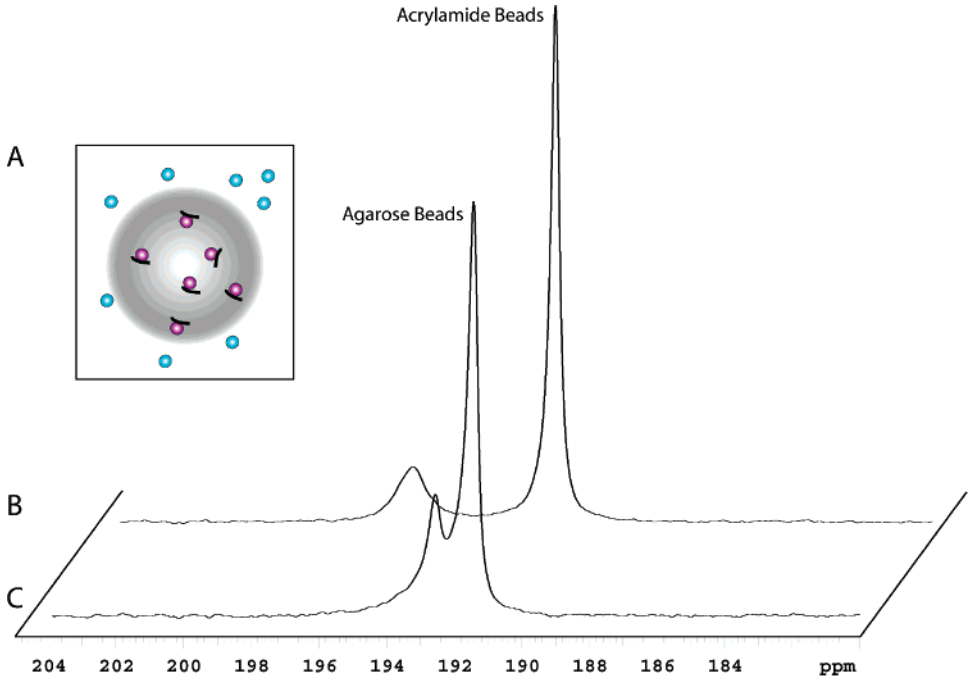

Figure 4. (A) Cartoon showing the two different environments that ^{129}Xe can nonspecifically probe. The small spheres illustrate the xenon atoms dissolved in solution and absorbed inside the bead matrix (large sphere). Expansions of the spectra around the xenon in solution peak are shown for acrylamide (B) and agarose (C) beads. The chemical shift of ^{129}Xe in solution is 192.3 ppm for both slurries, but the bead-associated ^{129}Xe chemical shifts are at 193.6 ppm for agarose and 195.4 ppm for acrylamide beads, reflecting a sensitivity of ^{129}Xe to the different bead environments.

and in turn the parameters for exchange signal averaging; the pool of xenon available for exchange may be different. Understanding all xenon resonances is important for characterizing the system and may be relevant in future experiments.

Although we have made progress in increasing the biosensor's sensitivity, limitations still exist. One specific limitation is the low concentration of the relevant spins (e.g., biosensor-bound ^{129}Xe) inside the sample volume, giving a low filling factor, weakening coupling of the spins to the coil. Such limitations due to the characteristics of NMR coil detection can be addressed in a fundamental manner by means of the recently developed NMR remote detection methodology.[15,35,36] The basic feature of this method is to separate the NMR frequency encoding and signal detection steps physically into two different NMR coils with separately optimized performance. With this approach, the resonance from the ^{129}Xe sensor can be encoded in the dilute sample environment of interest and then physically extracted, transferred, and concentrated into a much smaller detector volume to maximize the filling factor and, therefore, detection sensitivity. Our experiments demonstrate that ^{129}Xe can be introduced to and extracted from a liquid solution in a controlled manner. Coupling this technology with NMR remote detection is straightforward and promises further significant gains in sensitivity. For example, 75 nmol of xenon from 1.5 mL of sample solution could be extracted and concentrated as a pure gas into a volume of 0.15 μL (~1 atm). This represents a decrease in coil diameter from 10 mm to 100 μm, well within the range of commercially available microcoils for capillary NMR detection.[32,33] Therefore, remote detection could potentially allow for a filling factor increase of a factor of 10^4 and thereby lead to further sensitivity enhancement of 2 orders of magnitude.

CONCLUSIONS

We have presented a new technique that efficiently delivers LE ^{129}Xe gas into solution and have suggested the implementation of new methods for applying ^{129}Xe NMR biosensing to events in dilute aqueous and heterogeneous environments such as bead-immobilized cells.[37] We have demonstrated NMR biosensing in the nanomolar concentration range, which is by far the most dilute biomolecule concentration to be detected while retaining full NMR spectroscopic information. The possibility of accessing multiple sites and events in a mixture of different biomolecules suggests that the xenon biosensor may be useful in the characterization of heterogeneous materials and the detection of low-concentration metabolites in samples in vivo and miniaturized sensors.[38,39]

ACKNOWLEDGMENT

We thank Seth Rubin for scientific input when the origin of the idea was developed. This work was supported by the Director, Office of Science, Office of Basic Energy Sciences, Materials Sciences of the U.S. Department of Energy under Contract DE-AC03-76SF00098. S.-I.H. gratefully acknowledges the Alexander von Humboldt Foundation for support through a postdoctoral fellowship.

Received for review January 10, 2005. Accepted April 11, 2005.

AC0500479

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