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Ca^{2+} is a fundamental metal ion for physiological functioning. Therefore, molecular probes for Ca^{2+} analysis are required. Recently, a hyperpolarized NMR probe has emerged as a promising tool. Here, we report a new design of a hyperpolarized NMR probe for Ca^{2+}, which showed a large chemical shift change upon binding to Ca^{2+} and was applied for Ca^{2+} sensing under a hyperpolarized state.

In our body, various physiological functions are controlled by metal ions. One of the most important metal ions is Ca^{2+}, which plays many roles in our body. For example, some enzymes utilize Ca^{2+} as a co-factor to construct their elaborate structures.[1–3] Furthermore, the Ca^{2+} signalling pathway controls cellular functions.[4] On the other hand, the disruptions of Ca^{2+} homeostasis known as hypercalcaemia or hypocalcaemia are caused as a result of some diseases, such as chronic kidney disease or bone tumour.[5] Therefore, molecular probes for Ca^{2+} are required to elucidate the calcium-related physiological function.

To date, a number of fluorescent probes for Ca^{2+} have been developed.[6] These probes have contributed to a great number of studies and revealed the essential role of Ca^{2+}.

As a next-generation tool, nuclear magnetic resonance (NMR) molecular probes have attracted attention.[7,8] Compared with the fluorescent modality, the NMR signal, which utilizes radio waves, has high permeability in opaque samples. One example of an NMR probe for Ca^{2+} is 5F-BAPTA, developed by Rosen *et al.*,[9] which changes ^{19}F chemical shift upon binding to Ca^{2+}. Unfortunately, NMR probes typically suffer from low sensitivity.

Hyperpolarization, which can enhance the NMR signal, is a powerful technique to overcome this drawback. Recently, dynamic nuclear polarization,[10,11] parahydrogen-induced polarization,[12] and spin-exchange optical pumping have been utilized to polarize and dramatically enhance the signal from NMR probes. Hyperpolarized [1-^{13}C] pyruvic acid is a representative probe that reacts with lactate dehydrogenase (LDH) and is converted to [1-^{13}C] lactate, thereby reporting LDH activity.[13] Metabolic analysis with such hyperpolarized NMR probes has now become an active research field. However, the variety of hyperpolarized NMR probes is still limited. Little research has been done on hyperpolarized NMR probes for Ca^{2+}.[14,15]

Previously, we reported a hyperpolarized ^{15}N NMR probe targeting Ca^{2+} that utilized the [^{15}N,D_9]trimethylphenylammonium (TMPA) platform having an extraordinarily long ^{15}N hyperpolarization lifetime.[14] Although the designed [^{15}N,D_9]TMPA derivative worked as a chemical shift-switching probe, the ^{15}N chemical shift change upon binding to Ca^{2+} was small (1.5 ppm).

In general, large chemical shift changes are required for accurate analysis because a small difference suffers from difficulty in separating two signals. Such a chemical shift change is more crucial, especially for analysis in heterogeneous samples. Therefore, one of the key issues in this topic has been how to induce a large chemical shift change of hyperpolarized

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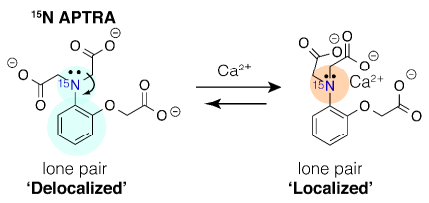
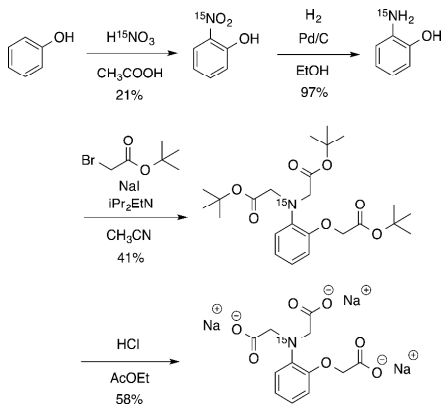


Fig. 1 The structure of ^{15}N APTRA and schematic illustration of the mechanism to induce a large ^{15}N chemical shift change upon binding of Ca^{2+}.



Scheme 1 Synthesis of ^{15}N APTRA.

nuclei upon binding or reaction with a target. In this study, we designed a new hyperpolarized ^{15}N NMR probe that induces a large chemical shift change upon binding to Ca^{2+}.

To this end, we focused on the electron density change of the ^{15}N atom in the Ca^{2+} chelator structure. The probe structure is based on *o*-aminophenol-*N,N,O*-triacetic acid (APTRA), which is known as a Ca^{2+} chelator, and labelled with a ^{15}N isotope as an NMR active atom (Fig. 1). A previous study has suggested the electron lone pair of aniline derivatives is normally delocalized into its aromatic ring, resulting in a large chemical shift change upon protonation ($\Delta\delta$: 4.9–25.1 ppm).[16] With this in mind, the lone pair of the ^{15}N atom in APTRA is expected to delocalize into the aromatic ring in the absence of Ca^{2+}, and be localized upon binding to Ca^{2+} because of the co-ordination. Therefore, we assumed that sufficient chemical shift change is induced in the ^{15}N of APTRA upon binding to Ca^{2+} (Fig. 1).

The ^{15}N labelled APTRA probe was synthesized from phenol in four steps (Scheme 1). ^{15}N labelled nitric acid was used as a ^{15}N source. Through nitration of phenol, ^{15}N was substituted onto the aromatic ring. Then, the $^{15}NO_2$ group was reduced to $^{15}NH_2$ *via* hydrogenation. Carboxymethyl groups were introduced by the reaction with *tert*-butyl bromoacetate. After deprotection by acid catalysis, ^{15}N APTRA was purified using gel permeation chromatography.

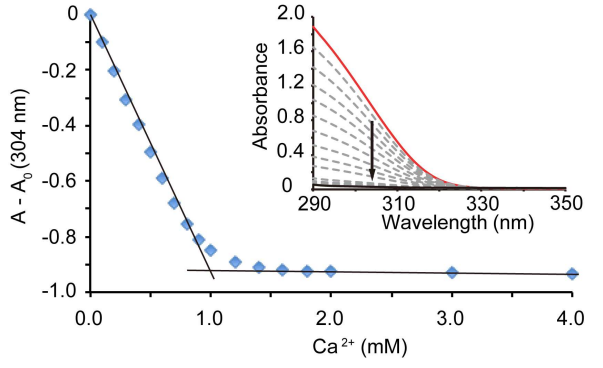


Fig. 2 Absorption spectral changes and titration plot obtained from absorption change at 304 nm, 1 mM ^{15}N APTRA, 20 mM HEPES buffer (pH = 7.4), 150 mM NaCl, 0–4 mM $CaCl_2$.

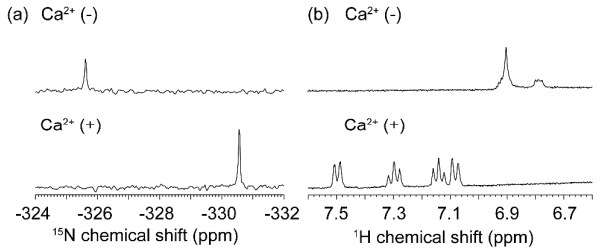


Fig. 3 Chemical shift change upon binding to Ca^{2+} (a) in ^{15}N NMR (768 scans, 90° pulse angle), (b) in ^{1}H NMR (128 scans, 45° pulse angle), 10 mM ^{15}N APTRA, 20 mM HEPES buffer (pH = 7.4), 150 mM NaCl, 10% D_2O, 0 or 20 mM $CaCl_2$.

We checked the Ca^{2+} binding ability of the probe. Ca^{2+} was added to a solution of ^{15}N APTRA (1 mM), and absorbance changes at 304 nm were measured. The titration plot indicated 1:1 binding for Ca^{2+} (Fig. 2). A higher concentration of ^{15}N APTRA (10 mM) also showed the same 1:1 binding (^{1}H NMR analysis, data not shown). A Job plot further supported these results (Fig. S1a). Dissociation constants (K_d) were determined using UV titration, giving 22 μM for Ca^{2+} and 1.9 mM for Mg^{2+} (20 mM HEPES pH = 7.4, 150 mM NaCl, Fig. S1c, e). The K_d values are similar to those of APTRA-based molecular probes.[17] These data indicate that the ^{15}N APTRA binds tightly to Ca^{2+} with a high selectivity over Mg^{2+}.

The Ca^{2+}-dependent ^{15}N chemical shift change was evaluated. In the ^{15}N NMR spectrum (Fig. 3a), a 5.0 ppm upfield shift was observed upon addition of 2 equiv of Ca^{2+}.[18] The observed shift is 3.3-times larger than that of the former hyperpolarized probe utilizing the [^{15}N,D_9]TMPA unit (1.5 ppm)[14] and is large enough to discriminate between these two peaks.

This upfield shift is consistent with our expectation. It is reasonable to think that the lone pair is localized upon binding to Ca^{2+}, resulting in high electron density around the ^{15}N atom. ^{1}H NMR data further support the mechanism. After addition of Ca^{2+}, the ^{1}H NMR spectrum showed a downfield shift of all ^{1}H signals in the aromatic region (Fig. 3b). This downfield shift is thought to be due to a decrease in electron density on the aromatic ring resulting from localization of the ^{15}N lone pair.

Finally, we performed a hyperpolarized NMR measurement. A ^{15}N APTRA solution (D_2O/DMSO-d_6 = 1:1) containing 15 mM OX63 was hyperpolarized at 1.4 K with irradiation at 94 GHz using a HyperSense (Oxford Instruments). The polarized sample was dissolved in 20 mM HEPES buffer (pH = 7.4) containing 150 mM NaCl and transferred to the NMR spectrometer (9.4 T) immediately. The hyperpolarized ^{15}N NMR signal was clearly observed by a single scan (Fig. 4a left, 25° pulse angle). Compared with the thermally equilibrated state (160 scans, 90° pulse angle, Fig. 4a right), the signal was enhanced about 4800 times.

Fig. 4b shows the time dependence of the hyperpolarized signal. The hyperpolarized signal could be observed for over 1 min under our experimental conditions (1 scan). The spin–lattice relaxation time (T_1) is known to correlate directly with the hyperpolarization lifetime. The T_1 value was calculated from the signal decay to be 37 s without Ca^{2+} and 36 s with 2 equiv of Ca^{2+} (3.3 mM ^{15}N APTRA, 20 mM HEPES buffer pH =

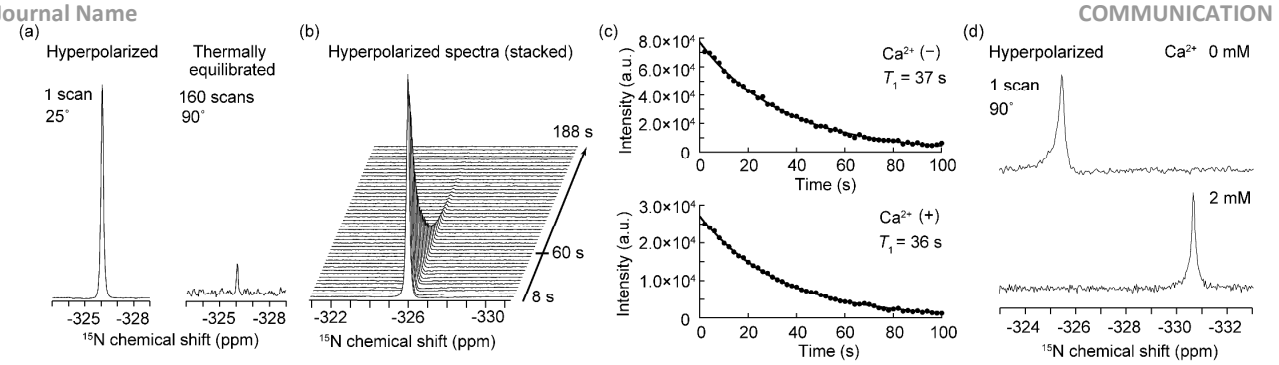


Fig. 4 (a) Hyperpolarized ^{15}N NMR signal of ^{15}N APTRA (9.8 mM) (1 scan, 25° pulse angle) and thermally equilibrated ^{15}N NMR signal of the same solution (160 scans, 90° pulse angle). (b) ^{15}N NMR spectra of hyperpolarized ^{15}N APTRA (9.8 mM) stacked from 8–188 s (every 4 s). (c) Signal decay of hyperpolarized ^{15}N APTRA (3.3 mM) without (top) or with (bottom) 2 equiv Ca^{2+}. (d) ^{15}N NMR spectra of hyperpolarized ^{15}N APTRA (0.97 mM) without or with 2 mM $CaCl_2$. Hyperpolarized samples were dissolved in 20 mM HEPES buffer (pH = 7.4) containing 150 mM NaCl (a, b, d) and 20 mM HEPES buffer (pH = 7.4) (c). Chemical shifts were calibrated using $^{15}NH_4Cl$ (–352.9 ppm) as an external reference.[19]

7.4, 9.4 T, Fig. 4c). These results indicate that Ca^{2+}-coordination hardly affects the T_1 value. Although the hyperpolarization lifetime of ^{15}N APTRA is shorter than that of the previous probe utilizing [^{15}N,D_9]TMPA (129 s, D_2O, 9.4 T), the lifetime was still longer than 30 s and is thought to be sufficient because the binding of Ca^{2+} is a very fast event (k_{on} = 2.39×10^8 M^{-1} s^{-1} in the case of 5F-APTRA[17]).

The ^{15}N APTRA achieved a large chemical shift change upon binding to Ca^{2+} in the hyperpolarized state. In the presence of 2 equiv of Ca^{2+}, the ^{15}N signal was observed at 5.2 ppm upfield position (Fig. 4d, spectra measured within 30 s after dissolution). The difference was almost the same as that obtained under a thermally equilibrated state. Importantly, and different from the result in Fig. 3a, the ^{15}N NMR analysis could be conducted with one scan because of the enhanced sensitivity of the hyperpolarized ^{15}N nuclei.

In summary, based on the lone-pair localizing mechanism, we designed hyperpolarized ^{15}N APTRA, which induced a large chemical shift change (approx. 5 ppm) upon Ca^{2+} binding and achieved Ca^{2+} sensing in a hyperpolarized state. To the best of our knowledge, the observed chemical shift change is the largest value among hyperpolarized ^{15}N NMR probes for metal ions. This large chemical shift would be an advantage for Ca^{2+} assays in biological samples.

The number of ^{15}N NMR probes is still limited. The present chemical shift-switching mechanism can be applied to design new hyperpolarized ^{15}N NMR probes targeting other metal ions or pH.

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