Multi-modal ^{19}F NMR probe using perfluorinated cubic silsesquioxane-coated silica nanoparticles for monitoring enzymatic activity†

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Received (in Cambridge, UK) 28th August 2008, Accepted 1st October 2008
First published as an Advance Article on the web 21st October 2008
DOI: 10.1039/b815022b

The establishment of ^{19}F NMR signal regulation and the application of this strategy to develop a multi-modal ^{19}F NMR probe for monitoring enzymatic activity using nanoparticles as a signal regulator is described; water-soluble perfluorinated cubic silsesquioxane was synthesized and immobilized onto the silica nanoparticles for suppressing the signals; ^{19}F NMR signals of the probes were recovered by releasing from nanoparticles.

Magnetic resonance imaging (MRI) is one of the powerful diagnostic tools as a noninvasive diagnosis method, and through the use of the contrast agents, site- and time-selective information can be received. ^{19}F MRI using fluorinated compounds as the contrast agents has recently gathered attention because the low level of endogenous fluorine leads to high signal to noise ratio in the images.[1] In addition, several groups have reported functional ^{19}F NMR probes which can detect enzymatic activity or environmental alteration by changing of their signal heights or chemical shifts.[2] Furthermore, fusion of multiple information has been increasingly required for improving the diagnosis accuracy, thus molecular imaging probes for the next generation should equip the multimodality.[3]

The accumulation of equivalent fluorine groups is essential for improving the sensitivity of the ^{19}F NMR signals, however, perfluorinated compounds exhibit extremely poor water-solubility. One way to solve these problems is to use cubic octameric polyhedral oligomeric silsesquioxanes (POSS) as a scaffold for the accumulation of fluorine atoms. POSS are highly-water-soluble nanoblocks, and it has been reported that POSS could form compact structure compared to the same generation of poly(amido)amine dendrimers.[4] These characteristics can be beneficial for the accumulation of the probe molecules with high density to improve sensitivity.

Herein, we report the regulation system of ^{19}F NMR signals for monitoring bioreactions based on water-soluble perfluorinated POSS-coated silica nanoparticles (NPs). The enzymatic reaction can be monitored by the enhancement of ^{19}F NMR signals. In addition, we develop the multi-modal probe with dual detection of fluorescence and ^{19}F NMR. This is the first example that the NPs can be used for NMR signal regulation.

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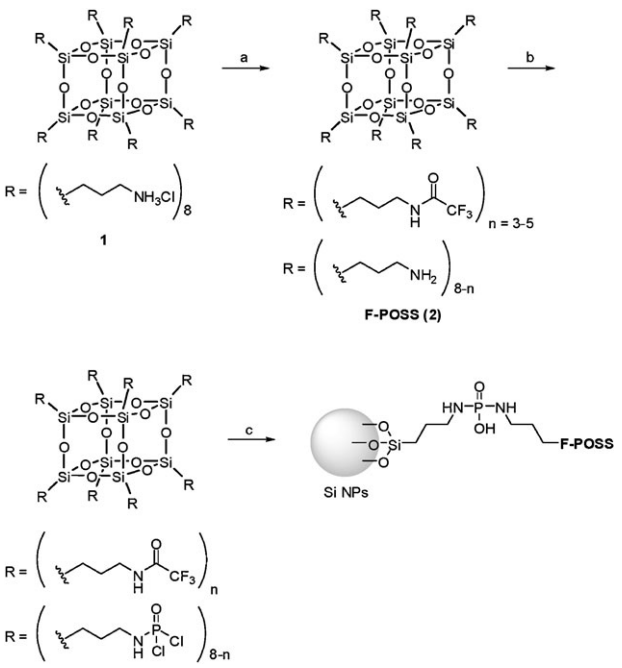
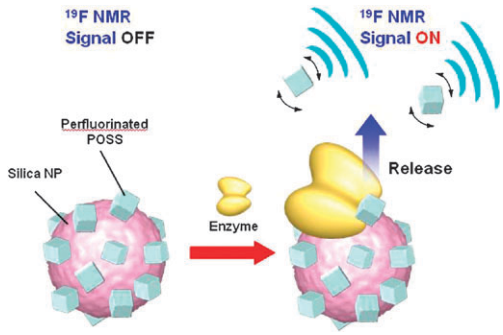
† Electronic supplementary information (ESI) available: Materials and methods and further characterising data. See DOI: 10.1039/b815022b

The chemical structures and synthesis of the water-soluble perfluorinated POSS (F-POSS) using octaammonium POSS **1**[5] as a starting material are shown in Scheme 1. F-POSS has trifluoroacetyl groups as the ^{19}F NMR signal moiety. The major product was identified with modified POSS containing POSS-TFA_4 from MALDI-TOF-MS measurements. The solubility of F-POSS in PBS (pH = 7.5) at 25 °C was at least 10 mM (120 mM F atom concentration), and significant peak broadening following the decrease of sensitivity was not observed by adding to BSA (1 mg mL^{-1}) or altering the pH between 5 and 9. In addition, the degradation of F-POSS was not observed after 24 h incubation at 37 °C at pH 7.0 in the presence of proteinase K. The detection limit was determined to be 10 μM using a ^{19}F NMR spectrometer with a 20 mm surface coil at 9.4 T. These results suggest that F-POSS could provide clear ^{19}F NMR signals without loss of sensitivity caused by unexpected interactions *in vivo*.

Scheme 2 illustrates the strategy for the regulation of ^{19}F NMR signals. In the solid state, the sensitivity of the NMR signal was decreased by the acceleration of the transverse relaxation time and the anisotropy of the spin toward the external magnetic fields. On silica NPs, the molecular rotation of the perfluorinated POSS should be highly restricted, and the NMR signals from the probe can be decreased.[2b] After releasing the probe triggered by an enzymatic reaction, the



Scheme 1



Scheme 2

NMR signals are recovered. Therefore, the enzyme activity can be detected by the enhancement of the signal intensity of ^{19}F NMR.

NPs in the range of 20 to 400 nm in diameter show tumor-selective integration known as the enhanced permeability and retention (EPR) effect.[6] In addition, the silica NPs can be attached with the fluorophores, and these fluorescent NPs displayed good feasibility for *in vivo* imaging because of high photo-stability and low toxicity.[7] We prepared amino-coated silica NPs averaging 150 and 280 nm in diameter containing or not-containing the fluorophores, respectively, using the Stöber method.[8] Amino groups on F-POSS were covalently linked to the amino groups on the silica NPs *via* the phosphordiamidate linker which can be cleaved by the enzymatic digestion of alkaline phosphatase (AP).[9] A class of AP is generally existing in the cell cytoplasm or surface in the whole body, and they can digest phosphodiester analogs without specificity.[9] Thus, in combination with the EPR effect, the monitoring system of the AP activity could be a new method for early cancer diagnosis.

The reaction mixture containing F-POSS and triethylamine in chloroform was added to phosphorus oxytrichloride at room temperature, and subsequently the amino-coated silica NPs were suspended in the mixture in one pot. The reaction was monitored by ninhydrin reagents for checking the consumption of the amino groups on the NPs. After purification with centrifuging and drying, we confirmed from dynamic light scattering that unexpected aggregation of the modified NPs was not found after modification with F-POSS, and the diameters of synthetic NPs were determined from the TEM images (see Fig. S1 in ESI†). From the fitting to the standard curve on ^{19}F NMR signal intensity, it was estimated that 100 ($\pm$20) nmol mg^{-1} of F-POSS was immobilized onto the silica NPs. Undesired decomposition of the probe did not occur by the proteinase K treatment or the pH alteration between pH 5 and 9.

The reactivity of the F-POSS-coated silica NPs with AP was investigated. Initially, the modified NPs averaging 150 nm in diameter were used. The reaction mixtures containing 3 mg mL^{-1} of the modified NPs (300 μM F-POSS) and AP in 50 mM sodium phosphate and 100 mM Tris-HCl buffer (pH = 7.0) were incubated at 37 °C, and ^{19}F NMR signals of the mixture were monitored (Fig. 1). The signal linearly increased until 12 h incubation (t_{50} = 6.2 h). In the absence of the enzyme, the NMR signal was hardly detected even after 24 h incubation. In the case of 280 nm diameter of the NPs, the rate

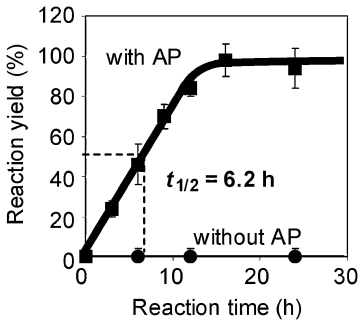


Fig. 1 Time-course of the enzymatic reaction. F-POSS-coated silica NPs (1.5 mg) were incubated in 500 μL of the reaction solutions containing AP (5 U) (■) and non-containing AP (●) in 50 mM sodium phosphate, 25 mM Tris-HCl, and 0.05 mM EDTA (pH = 7.0) at 37 °C. The reaction yields were monitored with ^{19}F NMR and calculated by the fitting on the standard curve.

of the signal intensity increase was of similar extent as those of 150 nm diameter particles (t_{50} = 6.0 h) (see Fig. S3 in ESI†). These results indicate that the ^{19}F NMR signals could be obtained independently of the size of NPs. The time-scale of these reactions might be adequate to obtain the EPR effect due to the prevention from non-specific digestion of the NPs during the transportation.

We demonstrated the multimodal detection with fluorescence and ^{19}F NMR for the enzymatic activity. The time course of ^{19}F NMR signals of the F-POSS-modified silica NPs containing fluorescein-5(6)-carboxylic acid is shown in Fig. 2(a) (quantitative data shown in Fig. S4 in ESI†). AP activity induced a signal enhancement after 3 h incubation, and the signal intensity was saturated after 12 h incubation. This result corresponds with the data shown in Fig. 1. Fluorescence emission from fluorescent silica NPs was constantly observed during reactions without photobleaching (Fig. 2(b)). These data suggest that the fluorescence can be used as a reference to zero for scanning the distribution of the probes and estimating the absolute intensity of the ^{19}F NMR signal.[10] Although the sensitivity of the probe for acquiring clear signals in the ^{19}F NMR spectra was lower than that for the fluorescence image, our system could have a possibility to

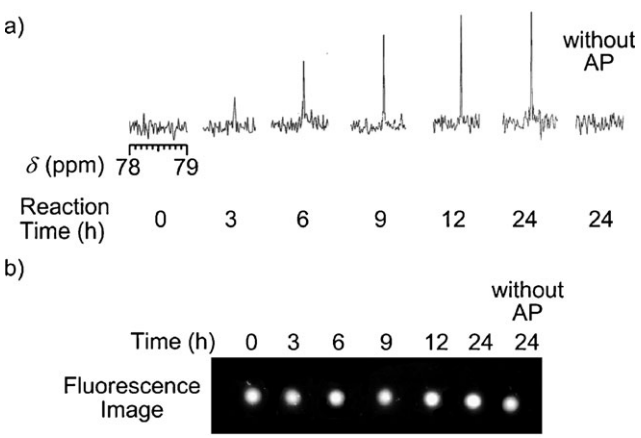


Fig. 2 (a) ^{19}F NMR spectra of F-POSS-coated silica NPs in diameter of 150 nm containing fluorescein-5(6)-carboxylic acid in enzymatic hydrolysis with AP. (b) The fluorescence image was taken using a transilluminator (365 nm) through a 480-nm long pass emission filter.

improve sensitivity by employing POSS-core dendrimers for incorporating a larger number of fluorine atoms into the probe.

In conclusion, we describe here a novel ^{19}F NMR probe using perfluorinated POSS, and the enzymatic activity can be monitored by the enhancement of their ^{19}F NMR signals. Using the fluorescent NPs, the dual detection with ^{19}F NMR and fluorescence was accomplished potentially for the simultaneous monitoring of the enzymatic activity and the biodistribution. Though there remains room to optimize reactivity or to evaluate the toxicity and the biodistribution in living animals, our system could be applied to the sensing of not only pH *via* hydrolysis but also other kinds of enzymes such as nucleases and proteases by modification with the linker. In addition, a detection modality can be added by the modulation of the material of NPs. It is expected that this strategy will be helpful for designing new generations of molecular imaging probes.

This study was conducted as a part of the project, "R&D of Molecular Imaging Equipment for Malignant Tumor Therapy Support," supported by NEDO (New Energy and Industrial Technology Development Organization). We thank Prof. Y. Tsuji and Dr T. Fujiwara for the measurement of ^{19}F NMR. We thank Prof. M. Nagao and Ms. M. Chujo for the MTT assay. We thank Prof. I. Hamachi and Mr. S. Fujishima for taking the fluorescence images.

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