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Novel Nuclear Magnetic Resonance Microcells for a Limited Volume

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ABSTRACT

Microcells were specially designed for a limited volume of macromolecules. A good homogeneity of horse cytochrome c (about 12 kD) is generated by Teflon insert. Glass plug filled with solvent exhibits a heterogeneity in the magnetic field. Wax filled tube effectively contributes the NMR signals. Splitting can be detected in good resolution in the microcells. Spin-spin interaction is also detetable. A minimium volume to get available NMR information for biological sample was demonstrated in this work. By using the novel microcell, wax in the bottom and Teflon plug on the top of the sample solution, the sample volume can be reduced down to six folds (about 75 μl) of the use in the standard tube.

Key words: Nuclear magnetic resonance, microcells, macromolecules, spin-spin interaction, splitting, limit volume, homogeneity

Nuclear magnetic resonance (NMR) has widely used for obtaining the three dimensional structure of the macromolecules such as DNA and proteins[1,2]. An NMR spectrometer provides the magnetic field to produce the energy levels, the ratio frequency to excite transitions, and a radiofrequency receiver to detect emitted radiation. The resolution of an NMR spectrum depends on the strength and homogeneity of the magnetic field and the constancy of the radiofrequency radiation.

Usually, it is both desirable and possible to observe species of protons in a concentration range well below 1 mM in biochemistry[3]. However, in aqueous solution, at least 0.1-1 M protons from H_2O interfere in the determining of sample molecules. To get good resolution, high concentration of sample solution is necessary except using high frequency NMR or water elimination[4]. For macromolecules, to prepare high concentration of sample solution have to use a large amount of sample. Several microcells for a limited amounts of samples are available on the markets; however, they place limitations on the experiments to the biological samples, for example bad resolution.

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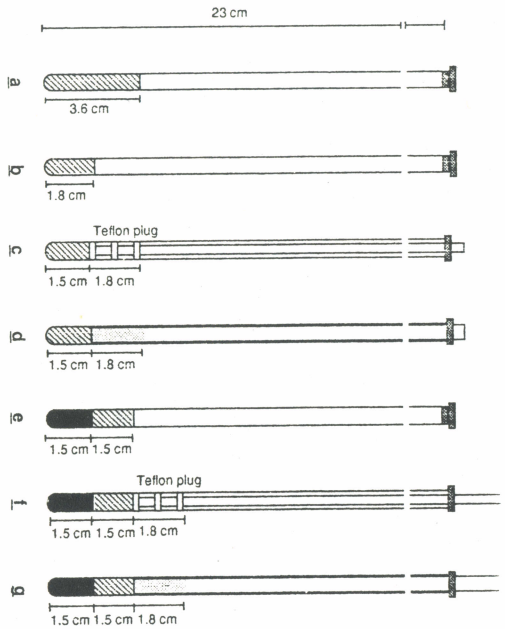

Fig. 1. An outline of a series of microcells specially designed for a limited amount of biological samples. $\underline{x}$ is assigned to represent different design described in the materials and methods. The height of sample is indicated by the side of the tube in centimeter scale. Sample solution (striped), wax (black), and an inner glass tube filled with D_2O (dot) are indicated. Teflon plug is marked beside the tube.

In this report, we proposed several types of microcells (figure 1) which specially designed for a limited volume of biological smaples. One of the main characteristic of these microcells are that they offer better resolution than that attainable with the standard tubes. In order to maintain the best resolution, the factors which may disturb the homogeneity in sample cell were excluded. To supress the generation of a vortex in sample cell was considered to control homogeneity. Two kinds of plugs, Teflon and glass, were inserted as vortex stopper. Considering the symmetric configuration around the detection coil, wax was used to fill the bottom of a standard tube. The splitting and spin-spin interaction were also determined. Finally, the minimium volume to get available NMR information has been demonstrated in the novel microcells.

MATERIALS AND METHODS

Horse heart ferric cytochrome c, DL-alanine, and nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Sigma Chemical Company. Proton nuclear magnetic resonance spectra were taken on a Varian XL-300 spectrometer.

Microcells specially designed for a limited amount of biological samples

Figure 1 shows an outline of a series of microcells: $\underline{a}$ & $\underline{b}$ are standard tubes filled with 500 μl (3.6 cm of height) and 250 μl (1.8 cm of height) of sample solution individually; $\underline{c}$ is an insertion type with Teflon plug on the top of the sample solution to supress the generation

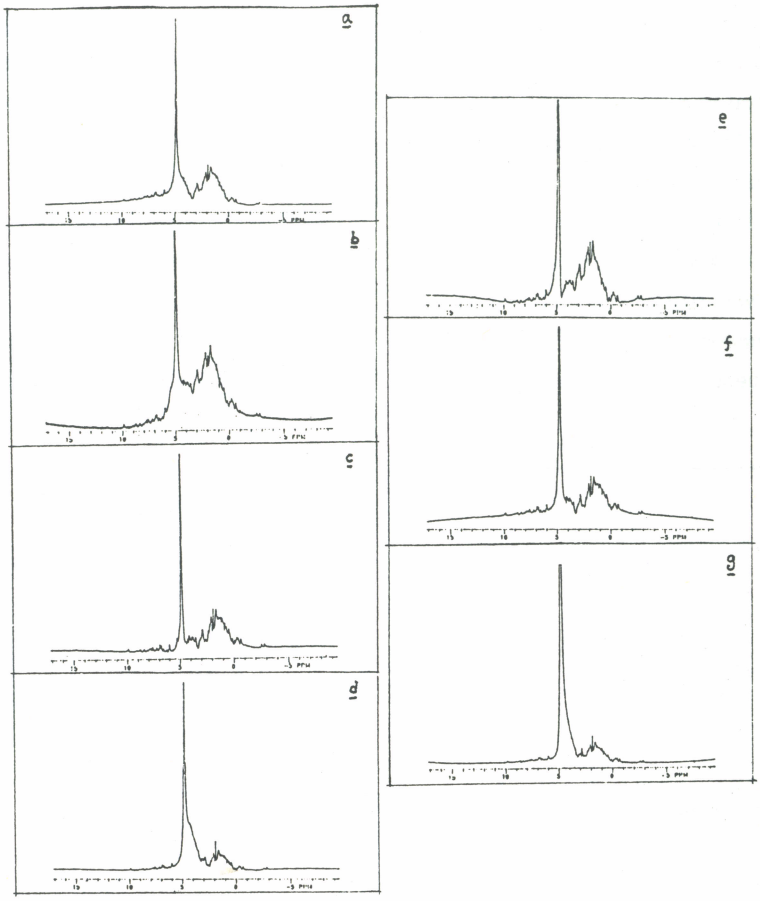

Fig. 2. The 300 MHz ^{1}H-NMR spectra of horse cytochrome c (about 4mM) obtained in microcells. x indicates the designs described in Figure 1.

of vortex; d is an insertion type with glass tube plug filled with the same solvent (D_2O) as utilized for sample solution for adjusting the magnetic susceptibility; f & g are the symmetric modification of c and d with wax filled in the bottom of the cells; e is control of f and g to observe the symmetric effect of wax. All of c, d, e, f, and g have the same height (1.5 cm) of sample solution.

RESULTS AND DISCUSSION

A good homogeneity is generated by Teflon insert

A macromolecule, cytochrome c (m. w. = 12, 384) was used to examine the different designs in the improvement of resolution. The 300 MHz ^{1}H-NMR spectra of horse heart ferric cytochrome c (about 4 mM) in the figure 2 are obtained from different experiments by using

different kinds of microcell, and x indicates the designs described in figure 1. As you can see, a is a standard spectrum. If we reduced the volume of sample to be a half, the peaks would become to be broad and not clear-cut, shown as b. The signal-to-noise became smaller. If we inserted the Teflon plug as a vortex stopper in tube b, the spectrum, c, became clear and much better than that of a, especially the peaks between 3.5 to 4.5 ppm. If replacing the Teflon plug with solvent (D_2O)-filled glass, the improvement was limited shown as d indicating the material influenced the resolution. Glass or D_2O has negative effect in field homogeneity and magnetic susceptibility. Wax in the bottom of tube, e, contributes the signals effectively to the NMR peaks compared to a. The peaks between 3.5 to 4.5 ppm got good resolution, but insensitive in the region of 0.5 to 1.5 ppm, which was the same as b. If we replaced the wax to be a container filled with D_2O in the bottom, there were broaden peaks and difficulty in shimming (data not shown). It is not clear to point what wax does. But, it seems that wax is inert in the magnetic field where glass or D_2O doesn't. If plus the design of c or d with that of e, there

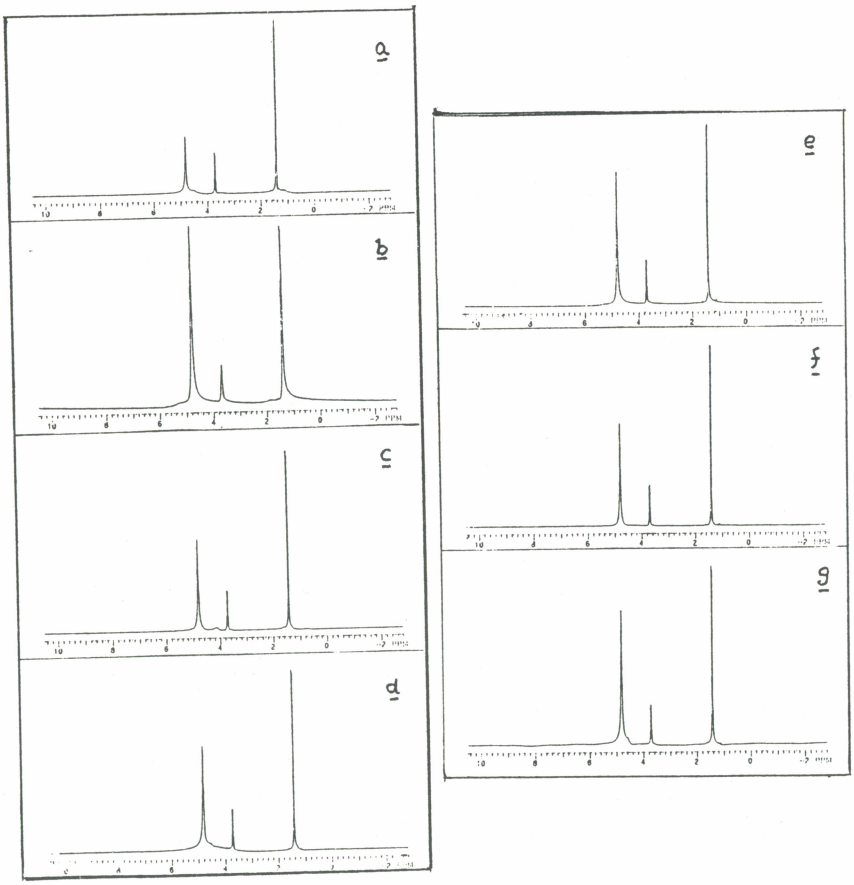

Fig. 3. The 300 MHz ^{1}H-NMR spectra of alanine (about 1M) obtained in microcells. x indicates the designs described in Figure 1.

is a good resolution in the f, but not quiet good in the g. The results of c and d suggest that the Teflon insert exactly brings a good field homogeneity and sample homogeneity. Previously, Takahashi and Nagayama[5] reported that a long glass insert made as a vortex stopper and filled with the same solvent can be utilized for sample solution to adjust the magnetic susceptibility; however, we found that both glass and D_2O cause the water peak broaden in d and g implying that the solvent in the sample solution and in the glass plug exhibit heterogeneity in the magnetic field. Our results also suggest that the solvent in the sample solution and in the glass plug exhibit heterogeneity in the magnetic field. In addition, they suggest that the volume of detected samples can be reduced down to 1.5 cm of height to get good resolution.

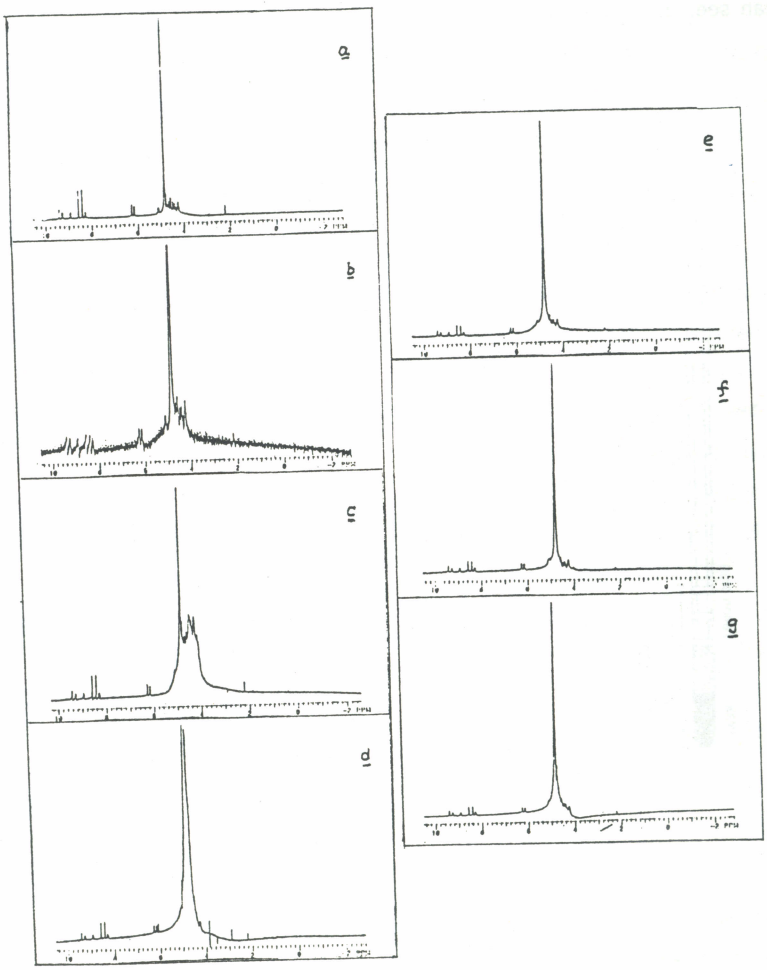

Fig. 4. The 300 MHz ^{1}H-NMR spectra of NADP (about 10mM) obtained in microcells. x indicates the designs described in Figure 1.

Splitting can be detected in good resolution in microcells

To examine if the microcells exhibit good splitting, we chose DL-alanine (m. w.=89.09) as a model molecule. DL-alanine was preapred in D_2O to be 1M. The purpose of high concentration is for easy and quick detection. The spectra would display highly sensitive plus a small water peak. In figure 3, the 300 MHz ^{1}H-NMR spectra contain a water peak at 4.8 ppm, a tetra-splitting peaks at 3.75 ppm and a displitting at 1.4 ppm, and x indicates the designs described in figure 1. As you can see, a is a standard spectrum. When reducing the sample volume in a half, it, b, didn't show good splitting. However, any of the designs of c, d, e, f & g can get same resolution as a. The results indicate that microcells specially designed for a limited amount of biological samples do not limit the resolution of splitting.

Spin-spin interaction is detetable in microcells

To examine if the microcells limit the spin-spin interaction, we chose nicotinamide adenine dinucleotide phosphate (NADP, m. w.=743.44) as a model molecule. NADP has a complicated

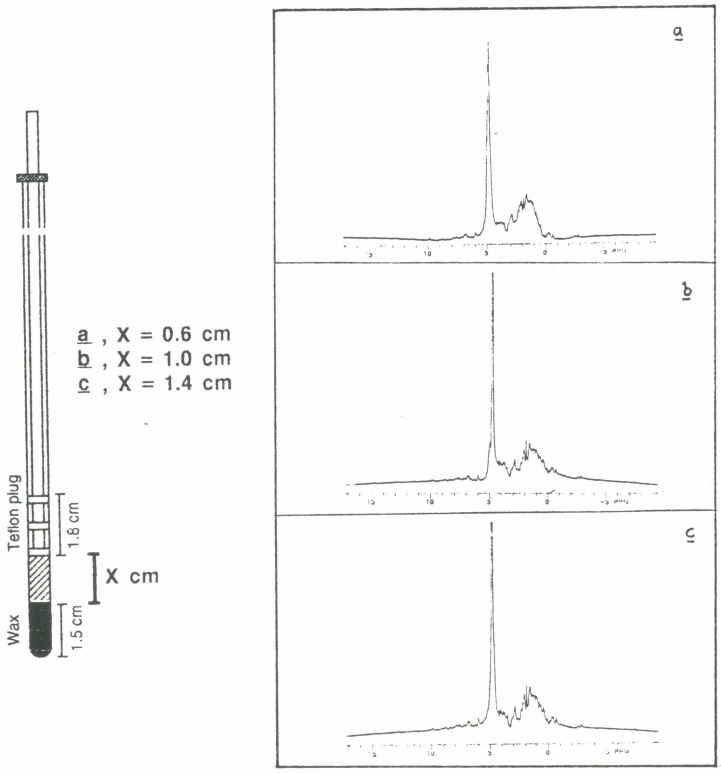

Fig. 5. Left, a special design as Figure 1 f: the sample volume can be adjusted by descending the Teflon plug; x indicates the height of the sample solution in centimeter scale; three kinds of volume are available in this experiment, i. e. a (0.6 cm), b (1.0 cm) and c (1.4 cm).

Right, the 300 MHz ^{1}H-NMR spectra of horse cytochrome c (about 4mM) obtained from a, b and c.

spectra because of spin-spin interaction. Ten mM NADP was preapred in D_2O for the experiment. The 300 MHz ^{1}H-NMR spectra show as figure 4, and $\underline{x}$ indicates the designs described in figure 1. As you can see, $\underline{a}$ is a standard spectrum. The S/N ratio is poor when the sample volume was reduced into a half shown as $\underline{b}$. Those designs, i. e. $\underline{d}$ and $\underline{g}$, improve the S/N ratio from $\underline{b}$; however, lossing the signal in the region of 4.2-4.7 ppm. Other designs, i. e. $\underline{c}$, $\underline{e}$ and $\underline{f}$, indicate that the water peak interferes the signs from the spin-spin interaction in the region of 4.2-4.7 ppm. The water peak can always cause trouble if your sample peaks locate near it. The potential interfering signals from water provide 110 M of protons if water is the solvent. Yet, in most cases, roughly 1 M total concentration from protein protons in ~1% protein solution, or 0.1-1M protons from residual water impurity in nominal deuterium oxide[3]. It is possible to overcome this problem when increasing the sample concentration.

Six folds reduction of sample volume still get the good resolution

What is the limitation of volume which is used to get good resolution? A special design has different capacity indicated in 0.6 cm, 1.0 cm and 1.4 cm of height of sample in the microcell shown as figure 5. Cytochrome c was used to examine the capacity limit in the same resolution. The 300 MHz ^{1}H-NMR spectra of cytochrome c (about 4 mM) show in figure 5 and $\underline{x}$ indicates the designs. The results illustrated a nice resolution in the height of 0.6 cm, $\underline{a}$, where sample is about 75 μl. A minimium volume to get available NMR information for biological sample was demonstrated in this work. By using the novel microcell 5 $\underline{a}$, the sample volume can be reduced down to 6 folds of the use in the standard tube.

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限量體積之核磁共振小池

陳建志　董一致

爲方便研究生物樣品，尤其是大分子，我們設計了一些限量體積之核磁共振小池。鐵氟龍插塞提供細胞色素 C(分子量約 12,000)很好的同質度。溶質填充的玻璃插塞表現了磁場的異質性。蠟填充之小池有效率的提供核磁共振的訊號。高解析度的訊號分裂性可以在這些限量體積之小池內測得。旋轉-旋轉交互作用也可測得。在這個實驗裡，核磁共振小池可提供在研究生物樣品時，得到可用的核磁共振資訊之最少體積。這個新的限量體積之核磁共振小池，在樣品液上方是鐵氟龍插塞，下方是蠟填充。使用這種小池可以減少樣品液體積至一般的六倍，大約是七十五微升。

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民國八十三年十二月二十四日受理