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Journal of Alloys and Compounds 408–412 (2006) 400–404

Journal of ALLOYS AND COMPOUNDS

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Study of rare earth encapsulated carbon nanomolecules for biomedical uses☆

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Received 30 July 2004; received in revised form 6 December 2004; accepted 6 December 2004
Available online 27 June 2005

Abstract

$Gd@C_{82}(OH)_{40}$ has been proposed to be as a new generation of the magnetic resonance imaging (MRI) contrast agent, but water-soluble fullerenols $Gd@C_{82}(OH)_n$ with $n > 36$ can easily lead to open-caged structures of a high instability. This restricts the practical bio-uses of Gd-metallofullerenols with a large number of hydroxyl groups. To explore how the imaging efficiency varies with decreasing hydroxyl number in $Gd@C_{82}(OH)_n$ of a good stability in vivo, $Gd@C_{82}(OH)_{22}$ was prepared, characterized and its imaging efficiency in mice was studied. This work aims at searching a chemical form of water-soluble Gd-metallofullerenols that satisfy both requirements of the good stability and high imaging efficiency in vivo. The results indicate that the proton relaxivity of $Gd@C_{82}(OH)_{22}$ is lower than that of $Gd@C_{82}(OH)_{40}$, but still higher than the commercial Gd-DTPA MRI contrast agent.

Keywords: Gd-metallofullerenol; $Gd@C_{82}(OH)_{22}$; Stability; MRI contrast agent; In vivo

1. Introduction

Probably, rare earth elements encapsulated metallofullerenes are the most important family in the endohedral fullerenes [1] (fullerene cage encapsulating atoms, clusters, or small molecules), because of their potential applications. So far, metallic elements that have been encapsulated by fullerene cages are shown in Table 1 (the bold element symbols). Among them, all of rare earth atoms encapsulated metallofullerenes can be produced by the conventional arc discharge method [2] similar to C_{60} production. Applications of these metallofullerenes are of great interest, and they have been proposed for potential applications in many fields including biomedical uses. More extensive applications come from properties of a tremendous number of their derivatives that can be designed according to the practical demands and produced by chemical/physical modifications of carbon cages.

Because of the unpaired electrons in the metallic atom of the rare earth element, their application in magnetic resonance imaging (MRI) is an intriguing topic. Shinohara et al. developed the first water-soluble gadolinium-metallofullerenes, $Gd@C_{82}(OH)_{40}$-based MRI contrast agent [3]. The measurement of water proton relaxivity (R_1) indicated that R_1 (the effect on the longitudinal relaxation rate, $1/T_1$) was about 20 times higher than that of the commercial MRI contrast agent Gd-DTPA in vitro. The MRI study of gadolinium fullerenol in mice confirmed its significantly

☆ A part of work was carried out in Beijing Synchrotron Radiation Facility.

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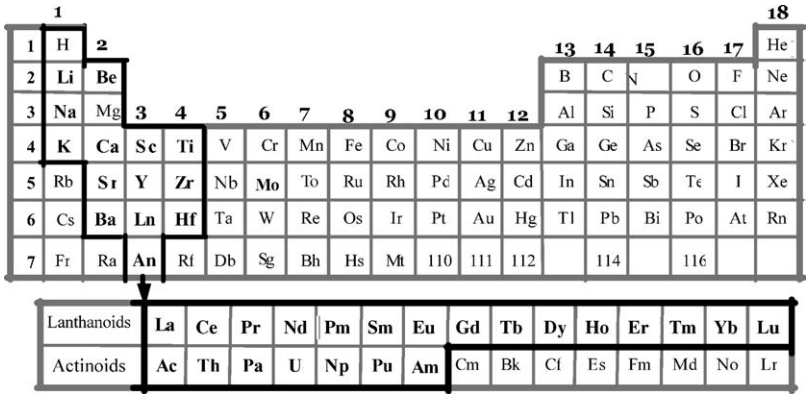
doi:10.1016/j.jallcom.2004.12.101

Table 1
All of the rare earth elements have been encapsulated by fullerene cages



In this periodic table, metallic elements marked by bold element symbols, some of their clusters, or small molecules have already been encapsulated into the fullerene cages, forming metallofullerenes. They are produced by the conventional arc discharge method similar to C_{60} production.

high imaging efficiency in vivo. It was hence proposed as a new generation of the MRI contrast agent. Recently, Bolskar et al. developed another water-soluble gadolinium-metallofullerenes, $Gd@C_{60}[C(COOH)_2]_{10}$ [4]. The relaxometry measurements reveal that it possesses a relaxivity comparable to that of commercially available Gd-DTPA-based MRI contrast agent.

As all these rare earth elements encapsulated carbon nanomolecules, $RE@C_{82}$, $RE@C_{60}$, etc., are not dissolved in water, they hence need to be water-solubilized for the biomedical uses. Previously, to gain better solubility required by practical applications, it was suggested to increase the number of the hydroxyl group. Recently, a study on the influences of structural properties on the stability of water-soluble fullerenes [5] found that the stability of fullerenols largely depends on the quantity of the attached exotic groups. In the synthesis process of fullerenol molecules, the coinstantaneous formation of impure groups was observed. It was found that the water-soluble fullerenols with hydroxyl group $n > 36$ can easily lead to open-caged structures, which are unstable and strongly influence functionalized properties mentioned above. Namely, we cannot use the water-soluble fullerenols with too many hydroxyl groups, in particular for applications in vivo. Accordingly, it is interesting to explore how the imaging efficiency varies with the decrease of the hydroxyl number of $Gd@C_{82}(OH)_n$. To assure the good stability of MRI contrast agent in vivo, one may have to reduce the hydroxyl number of $Gd@C_{82}(OH)_n$ in practical uses. To this end, we prepared water-soluble Gd-fullerenols with a less number ($n \sim 22$) of the hydroxyl groups (about half compared to $Gd@C_{82}(OH)_{40}$ [3]), and examined its imaging efficiency in mice. The work aims at searching an appropriate chemical form for water-soluble Gd-fullerenols that satisfies both conditions of good stability and high efficiency for magnetic resonance imaging in vivo.

2. Experimental

The metallofullerenes were synthesized using arc discharge method [2]. The soot was dissolved in DMF, and the products were extracted using a high-temperature and high-pressure method [6]. Separation and isolation of $Gd@C_{82}$ were performed using the high-performance liquid chromatography (HPLC, LC908-C60, Japan Analytical Industry Co.) coupling with 5PBB and then Buckyprep columns (Nacalai Co., Japan) [7]. The isolated $Gd@C_{82}$ species were identified by the matrix-assisted laser desorption time-of-flight mass spectrometer (MADLI-TOF-MS, AutoFlex, Bruker Co., Germany). The purity of the final $Gd@C_{82}$ product was about 99.5%. The synthesis method of water-soluble Gd-fullerenols was the alkaline reaction [3,8]. The $Gd@C_{82}$ toluene solution was first mixed with aqueous solution containing 50% NaOH, and then several drops of catalyst of 40% tetrabutylammonium hydroxide (TBAH) were added into the reaction system. The mixture of solutions was vigorously stirred at room temperature, the color of the solution in beaker was changed from the originally deep violet into colorless, meanwhile a brown sludge precipitated onto bottom of the beaker. After adding more water into the brown sludge, it was stirring over night. The brown precipitate was washed using MeOH, which was then removed by the vacuum-evaporation system. This washing manipulation was repeated several times for a complete removal of the remnant TBAH and NaOH. Finally, the brown precipitate was dissolved into deionized water with continuous stirring for 24 h until the solution color became a clear reddish brown. Then, it was purified by a Sephadex G-25 column chromatography (5 cm × 50 cm) with an eluent of neutralized water. The remained trace catalyst and Na^+ ions were completely removed in this process. To obtain a final Gd-metallofullerenol product of a narrow region of distribution of

the hydroxyl number, the fraction (eluate) was collected in a time interval of only several minutes. The Elemental analysis method was used to measure the number of hydroxyl groups. Due to the fact that a precise determination of hydroxyl number is important for the present study, a further measurement of the hydroxyl number was performed using X-ray photoemission spectroscopy. The samples used in XPS experiment were deposited onto the high-purity golden substrates to obtain thin films for the XPS measurements, which were carried out at ultra vacuum chamber with background pressure of $\sim 8 \times 10^{-10}$ Torr, and $\sim 1 \times 10^{-9}$ Torr during the measurement. The photon with energy $h\nu = 400.0$ eV from synchrotron radiation was used as the excitation source. The experimental energy resolution was estimated to be $\sim$0.5 eV. To inspect the contamination, XPS survey scans on the surface were performed before and after measurements. Then, the bioactivity of the water-soluble gadolinium-metallofullerene in living mice was studied by the magnetic resonance imaging technique. KM mice ($\sim$20 g, female, two months) were injected with $Gd@C_{82}(OH)_{22}$ via subaortic caudal vein. This was carried out in compliance with the national regulation about animal experiments. The injection dose was 6.5 μmol Gd/kg (1/20 of the typical clinical dose of Gd-DTPA, the commercial MRI contrast agent). After a single i.v. injection, the bioactivities of $Gd@C_{82}(OH)_{22}$ in mice were investigated using a Bruker 4.7 T/30 cm Biospec magnetic resonance imaging scanner.

3. Results and discussion

Fig. 1 shows the chromatographic spectra of 5PBB column. The peak at about 60 min corresponds to the species of $Gd@C_{82}$. Further isolation was carried out with Buckyprep columns, the chromatogram is displayed in Fig. 2. The HPLC-isolated $Gd@C_{82}$ was identified using MALDI-TOF-MS. The mass spectrum of the pure $Gd@C_{82}$ is shown in Fig. 3. The observed and theoretical masses for $Gd@C_{82}$ are $m/z = 1141.8$ and 1141.9, respectively. $Gd@C_{82}$ with a high purity of greater than 99.5% was achieved. After the hydroxylation of $Gd@C_{82}$, the element analysis was

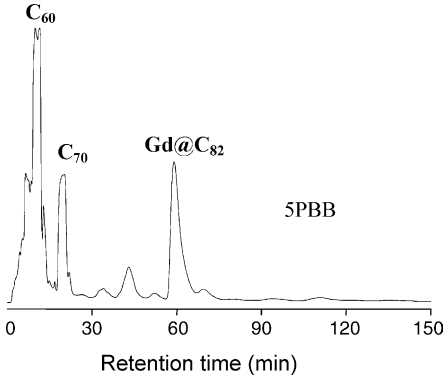


Fig. 1. The HPLC chromatogram for $Gd@C_{82}$ in 5PBB column.

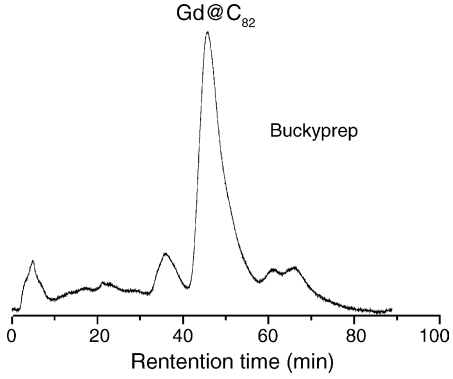


Fig. 2. The HPLC chromatogram for $Gd@C_{82}$ isolation in Buckyprep column.

employed to determine the hydroxyl number of the final products $Gd@C_{82}(OH)_n$, and gave $n \sim 24$. As it is known that the EA method is not a precise means for determining the hydroxyl number. We hence tried to analyze $Gd@C_{82}(OH)_n$ using MALDI-TOF-MS technique, but it is quite difficult to observe the mass peak of molecular ions. Comparing with $Gd@C_{82}$ molecule, $Gd@C_{82}(OH)_n$ shows a tendency of more easy fragmentation. This indicates that the stability of hydroxylated Gd-metallofullerene $Gd@C_{82}(OH)_n$ is somewhat declined compared to $Gd@C_{82}$ of non-hydroxylation.

To obtain a more precise result of the hydroxyl number, we used the X-ray photoemission spectroscopy (XPS) to measure the binding energy spectra of C 1s electrons for C–C and C–O bonds in the $Gd@C_{82}(OH)_n$ molecule. Fig. 4 shows the results, the binary structure of C 1s XPS spectra. For the pure component of C 1s electrons, the XPS spectrum should be symmetric and well described by a true Voigt function with a Gaussian dispersion [9]. The Gaussian analysis of the measured XPS data for $Gd@C_{82}(OH)_n$, as indicated in Fig. 4, exhibits at least two components: one centred around 284.9 eV is the C 1s binding energies of sp^2 non-functionalized carbons (C–C), in good agreement with

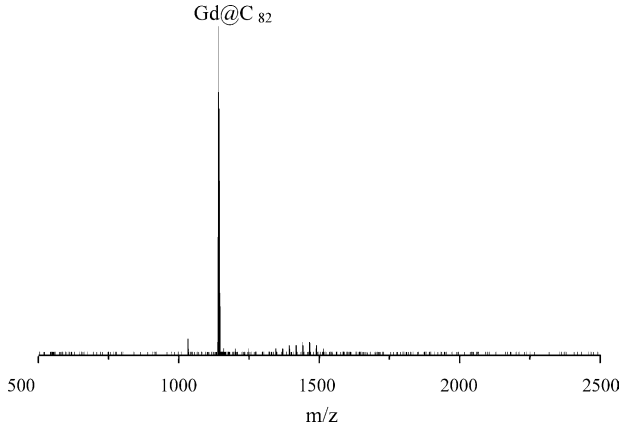


Fig. 3. MALDI-TOF mass spectrum for the isolated $Gd@C_{82}$. In a wide mass region from 500 to 2500, there are no other species being observed. The observed and theoretical masses for $Gd@C_{82}$ are $m/z = 1141.8$ and 1141.9, respectively. The purity of $Gd@C_{82}$ is estimated to be $\sim$99.5%.

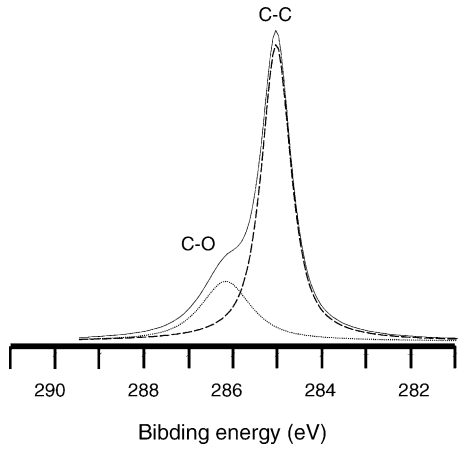


Fig. 4. The C 1s XPS spectra for Gd@$C_{82}(OH)_{22}$. The spectra for sp^2 non-functionalized carbons (C=C) and the hydroxylated carbons (C–OH) are indicated using broken and dotted lines, respectively. The number of the latter should be approximately equal to the number of the hydroxyl.

the value observed from C_{60} [9]; the other centred around 286.1 eV is for hydroxylated carbons (C–OH).

As the XPS spectra can differentiate the different carbons in Gd@$C_{82}(OH)_n$ molecule, this provide us with a more precise method to determine the hydroxyl number in Gd@$C_{82}(OH)_n$ based on the intensities for the non-functionalized and hydroxylated carbons. The intensities of C 1s components for non-functionalized and hydroxylated carbons in Gd@$C_{82}(OH)_n$ were estimated from integration of the corresponding peak areas under the dot line (hydroxylated carbons, C–O) and the broken line (non-functionalized carbons, C–C) in Fig. 4, respectively. As the total number of carbons is known to be 82, hence from the intensity ratio of sp^2 non-functionalized and hydroxylated carbons (by normalizing them to the total area under the solid curve), we can calculate the number of hydroxylated carbons that are just the number of n. It is ∼21, smaller than $n \sim 24$ obtained by EA. Taking into account of the ambiguity in both analysis methods, n was finally determined to be 22 ± 2. Thus, the chemical form of Gd-metallofullerenol used for the present MRI imaging experiment in vivo is Gd@$C_{82}(OH)_{22}$.

Fig. 5 shows typical images for kidney of mice. It is the T_1-weighted image after a single i.v. administration of Gd@$C_{82}(OH)_{22}$ at a dose of 6.5 μmol Gd/kg. The portion marked with number 2, 3, . . ., and 8 corresponding to the pictures of the image taken at the different time post injection (1 is the image before the injection of Gd@$C_{82}(OH)_{22}$). The brightness of kidney indicates that at an OH-number of as low as 22, the imaging efficiency is still high. In [3], Shinohara and co-workers studied the Gd@$C_{82}(OH)_{40}$ for MRI contrast agent and found the highest relaxivity. The relaxivity is mainly dominated by the exchange rate of protons between the water molecules surrounding the paramagnetic centre and those in the tissue. Unlike in the commercial MRI contrast agent Gd-DTPA, Gd^{3+} is encaged by a closed carbon cage in Gd@$C_{82}(OH)_n$, the exchange of water protons takes place only through the H of the hydroxyl group bound with the outer surface of carbon cage. Generally speaking, the larger the hydroxyl number is, the higher the relaxivity should be. This is in good agreement with the present observation that the proton relaxivity of Gd@$C_{82}(OH)_{22}$ is lower than that of Gd@$C_{82}(OH)_{40}$ [3].

Recently, Xing et al. [5] found that the coinstantaneous formations of highly oxygenated carbons such as ketone structure (impure groups) in the fullerene cage could not be turned away from formation processes of fullerenols, even if they were prepared using the alkaline reaction instead of strong acidic or oxidative processes. In a further study on the correlation between the stability and structure of fullerenols, they found that the degree of dissociation of the fullerenol

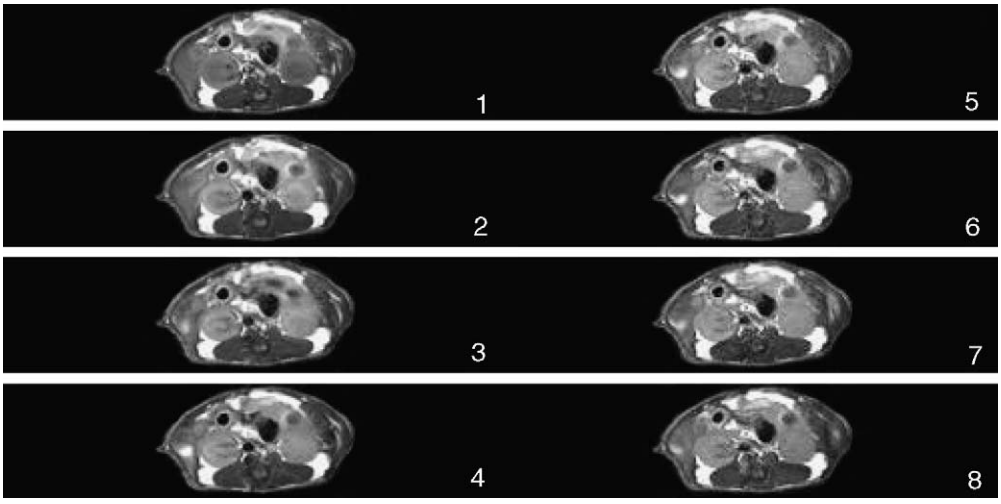


Fig. 5. Representative in vivo MR images focusing on a cross-section containing kidneys. Pictures marked by 1 is the image before administration, while those marked by numbers 2–8 correspond to the images taken at 0, 9, 18, 27, 35, 44 and 52 min after administration of Gd@$C_{82}(OH)_{22}$.

directly depended on the intensity of impure groups in the cage of fullerenols, especially the highly oxygenated carbons that led to a cage-opened structure and hence largely lowered the stability of the fullerenol molecule. The intensity of highly oxygenated carbons shows an increasing feature as the number of hydroxyl increases. A conventional consideration is to increase the number of hydroxyl groups to gain a better solubility in practical applications, but the break of the cage will lead to the release of Gd^{3+} ions which are highly toxic in vivo. In order to satisfy a good biostability of the Gd-metallofullerenol, practically, the number of hydroxyl cannot be too large. Compared with $Gd@C_{82}(OH)_{40}$, the hydroxyl number is only about half in $Gd@C_{82}(OH)_{22}$ which is a highly stable chemical form, yet, it still possesses a higher proton relaxivity than the commercial Gd-DTPA MRI contrast agent.

In summary, when $Gd@C_{82}(OH)_n$ of a large hydroxyl number (n) is used as the MRI contrast agent, it easily forms unstable open-caged structure, hence leading to an unexpected release of highly toxic Gd^{3+} ions in vivo. This restricts the biomedical applications of Gd-metallofullerenols of a large hydroxyl number. $Gd@C_{82}(OH)_n$ having a smaller n and a higher stability are practically required. We synthesized and characterized $Gd@C_{82}(OH)_n$ with $n = 22 \pm 2$, explored if it possesses an adequate imaging efficiency for MRI uses. In vivo MRI experiment showed that the proton relaxivity of $Gd@C_{82}(OH)_{22}$ is lower than that of $Gd@C_{82}(OH)_{40}$, but still higher than Gd-DTPA currently used in clinic. This suggests that the Gd-metallofullerenols with a much smaller OH-number can be also served as the new generation of MRI contrast agent.

Acknowledgements

We thank Prof. Yuanfang Liu of Peking University for discussion. Authors acknowledge the fund supports from Ministry of Science & Technology (2001CCA03800), National Natural Science Foundation of China (10490180), Chinese Academy of Sciences, National Centre for Nanoscience and Nanotechnology China (90406024), and State Key Laboratory of spectroscopy and Atomic and Molecular Physics (T152302).

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