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## ARTICLE

## The Effect of hemiketals on the Relaxivity of Endohedral Gadofullerenols

Cite this: DOI: 10.1039/x0xx00000x

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Received 00th January 2012,  
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Gadofullerenols has received much attention due to high relaxivities and low-toxicity as magnetic resonance imaging (MRI) contrast agents. However, the relaxivities of gadofullerenols vary from different reports. In this study, Gd@C<sub>82</sub>(OH)<sub>x</sub>O<sub>y</sub> with different hemiketal contents were synthesized and the influence on water proton relaxation was investigated. The results show that the Gd@C<sub>82</sub>(OH)<sub>x</sub>O<sub>y</sub> with more hemiketals exhibits much higher relaxivities than that with less hemiketals. The hemiketal structure is supposed to promote the formation of intermolecular hydrogen bond, and finally lead to the large aggregates. This is consistent with the previous report that the relaxivities of endohedral gadofullerene derivatives are greatly associated with the aggregate size. Hence, this work provides an important strategy to manipulate the relaxivity of gadofullerenes based MRI contrast agents by controlling the quantity of hemiketals on carbon cage.

### Introduction

Endohedral metallofullerenes have been extensively investigated in many medical applications, such as anti-tumor<sup>[1-3]</sup>, radiotracers<sup>[4,5]</sup>, X-ray diffraction contrast agents<sup>[6]</sup> and magnetic resonance imaging (MRI) contrast agents<sup>[7-10]</sup>. As MRI contrast, water-soluble gadofullerene derivatives possess much higher relaxivity than Magnevist and Ominiscan in clinical diagnosis<sup>[11-13]</sup> due to their abundantly exchangeable protons and proximity of water molecules to the paramagnetic center of gadofullerenes<sup>[14-15]</sup>. Moreover, gadofullerenes display much lower toxicity than gadolinium (III) chelate compounds, such as Gd-DTPA and Gd-DO3A, since the Gd<sup>3+</sup> ion is well protected by the fullerene cages. In addition, the easy modification of carbon cage makes gadofullerenes multifunctional<sup>[16-19]</sup>. These combined superiorities make water-soluble gadofullerene derivatives promising as the new generation of MRI contrast agents.

However, the gadofullerenes show diversity of relaxivities in fact. Primarily, the relaxivities of gadofullerenes strongly depend on the introduced functional moieties. Because of the poor water solubility, pristine gadofullerenes usually are modified with hydrophilic groups (commonly hydroxyl, carboxylate and sulfonate et al) before medical applications. As a result, different water proton relaxations were obtained

according to the different functionalization. For example, hydroxyl functionalized gadofullerenes show significantly higher relaxivities than that of carboxylic derivatives<sup>[20-21]</sup>, such as 38.5 mM<sup>-1</sup>s<sup>-1</sup> for Gd@C<sub>60</sub>(OH)<sub>x</sub> and a maximum relaxivity of 10.4 mM<sup>-1</sup>s<sup>-1</sup> for Gd@C<sub>60</sub>[C(COOH)<sub>2</sub>]<sub>10</sub> at high magnetic fields (30-60 MHz)<sup>[21]</sup>. Furthermore, Laus et al.<sup>[20]</sup> proved that the aggregating size of endohedral metallofullerenols also played predominant role in proton relaxation. In high concentration of PBS solution that can destroy the hydrogen-bonding of gadofullerene aggregates, the relaxivities of gadofullerenols decreased from more than 80 mM<sup>-1</sup>s<sup>-1</sup> to approximately 10 mM<sup>-1</sup>s<sup>-1</sup> at 60 MHz, and finally equal to that of carboxyl derivatives. Thus, to obtain the controllable relaxivity, it is necessary to further explore more parameters determining the relaxivities of gadofullerenes.

Gadofullerenols Gd@C<sub>82</sub>(OH)<sub>x</sub>O<sub>y</sub>, in which “x” and “y” refers to the number of hydroxyl and hemiketal respectively, is presently the most widely used water-soluble derivatives among gadofullerene based MRI contrast agents due to its simple preparation and high relaxivities. However, the relaxivities of Gd@C<sub>82</sub>(OH)<sub>x</sub>O<sub>y</sub> vary greatly from different reports<sup>[20]</sup>, but the reason is far from satisfaction. Herein, gadofullerenols with different hemiketal contents were synthesized by either alkaline reaction (more hemiketals)<sup>[13,22-</sup>

<sup>23]</sup> or hydrolysis of polycyclosulfated precursors (less hemiketals)<sup>[24-25]</sup>. And then the water proton relaxations were evaluated at 0.5 T to study the influence of hemiketals on the relaxivity of Gd@C<sub>82</sub>(OH)<sub>x</sub>O<sub>y</sub>. Although various proportions of hemiketals have been reported to lead to the diversities of biological response of fullerenols<sup>[26-32]</sup>, as far as we know, there is no report regarding the effects of the hemiketal structures on the properties of Gd@C<sub>82</sub>(OH)<sub>x</sub>O<sub>y</sub>, which may provide more insight into the relationship between molecular structure and relaxivity of gadofullerenols.

## Experimental

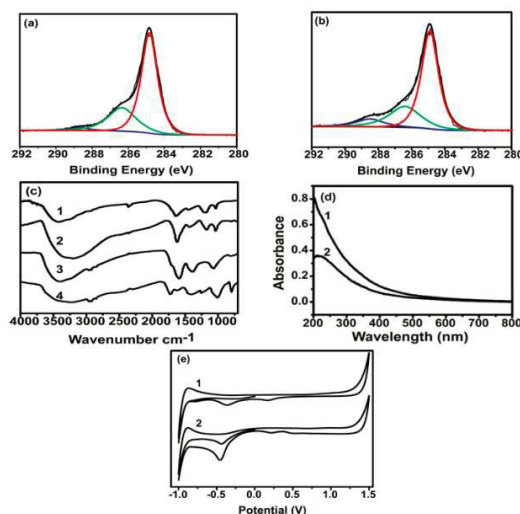
### Materials and methods

The reagents and organic solvents were purchased from commercial sources and used as received. Gd@C<sub>82</sub>(OH)<sub>x</sub>O<sub>y</sub> were prepared according to the literatures, such as alkaline reaction<sup>[22]</sup> and hydrolysis of polycyclosulfated precursors<sup>[24]</sup>. UV-vis absorption spectra were measured on a UNIC UV-4802H spectrometer in aqueous solution at room temperature. The Gd<sup>3+</sup> concentrations were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES, ICP-9000, Shimadzu, Japan). X-ray photoelectron spectroscopy (XPS) was performed on the Thermo Scientific ESCALab 250Xi using 200 W monochromated Al K $\alpha$  radiation. Fourier transform infrared (FTIR) spectroscopy measurements were recorded on a Nicolet Magna-IR750 spectrometer. The relaxation times were measured at 0.5 T with NMI20 Analyst instrument (Shanghai Niumag Corporation, Shanghai, China) and 7 T MR systems (BioSpec70/20USR, Bruker, Germany). The inversion-recovery method was used to measure  $T_1$ . The relaxivities  $r_1$  in either pure water or saline were obtained from the slopes of the relaxation time ( $T_1$ ) vs the Gd<sup>3+</sup> concentration, respectively. Dynamic light scattering (DLS) measurements were carried out with a Malvern Instrument (Zetasizer/nano Series, model ZEN3600). Scanning electron microscope (SEM) images were carried out on a JEOL JSM-6701F electron microscope. Transmission electron microscopy (TEM) images were performed using a JEOL JEM-2100F microscope. Cyclic voltammetry (CV) was recorded on an electrochemical workstation (CHI660D). Pt and Ag/AgCl were used as auxiliary electrode and reference electrode, respectively, and 0.5 M KNO<sub>3</sub> aqueous solution was served as supporting electrolyte. The scan rate was 100 mVs<sup>-1</sup>.

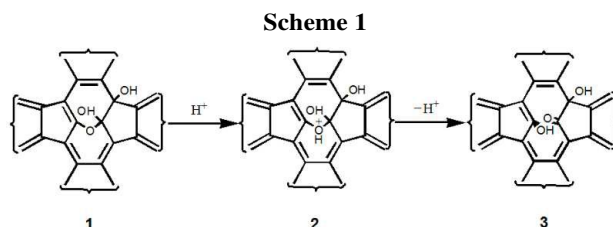
## Results and discussion

Gd@C<sub>82</sub>(OH)<sub>-20</sub>O<sub>-2</sub> and Gd@C<sub>82</sub>(OH)<sub>-21</sub>O<sub>-7</sub> were prepared by hydrolysis of polycyclosulfated precursors, and alkaline reaction in the presence of tetrabutylammonium hydroxide (TBAH), respectively. The structures were estimated by XPS (Fig. 1a and b), which is a preferred tool that has been extensively applied to evaluate the structure of water-soluble fullerene derivatives<sup>[18-19, 33]</sup>. Different carbons in carbon cage have different binding energy. The peak around 284.9 eV belongs to the non-functionalized carbons (C-C), the peaks at 286.6 eV and 288.6 eV are attributed to C-OH and C=O, respectively<sup>[22, 26]</sup>. The average number of hydroxyl and hemiketal (RO-C-OH) groups on carbon cage could be estimated as Gd@C<sub>82</sub>(OH)<sub>-20</sub>O<sub>-2</sub> and Gd@C<sub>82</sub>(OH)<sub>-21</sub>O<sub>-7</sub> for the production of hydrolysis of polycyclosulfated precursors and alkaline reaction, respectively, according to the C:O atomic ratio and the different chemical states for carbon. Although the

prepared Gd@C<sub>82</sub>(OH)<sub>-20</sub>O<sub>-2</sub> and Gd@C<sub>82</sub>(OH)<sub>-21</sub>O<sub>-7</sub> possessed similar structure and comparative hydroxyl number, the contents of hemiketal groups are significantly different. This is in well accordance with the previous report that fullerenols prepared by hydrolysis of polycyclosulfated precursors disfavor hemiketal structure<sup>[24]</sup>.



**Fig. 1** C1s fitting XPS spectra of Gd@C<sub>82</sub>(OH)<sub>-20</sub>O<sub>-2</sub> (a) and Gd@C<sub>82</sub>(OH)<sub>-21</sub>O<sub>-7</sub> (b); FTIR spectra of Gd@C<sub>82</sub>(OH)<sub>-20</sub>O<sub>-2</sub> (1#), Gd@C<sub>82</sub>(OH)<sub>-20</sub>O<sub>-2</sub> treated with HCl (2#), Gd@C<sub>82</sub>(OH)<sub>-21</sub>O<sub>-7</sub> (3#), Gd@C<sub>82</sub>(OH)<sub>-21</sub>O<sub>-7</sub> treated with HCl (4#) (c); UV-vis spectra of Gd@C<sub>82</sub>(OH)<sub>-20</sub>O<sub>-2</sub> (1#) and Gd@C<sub>82</sub>(OH)<sub>-21</sub>O<sub>-7</sub> (2#) in aqueous solution (d). Concentration: 5 × 10<sup>-6</sup> mmol/L; Cyclic voltammogram of Gd@C<sub>82</sub>(OH)<sub>-20</sub>O<sub>-2</sub> (1#) and Gd@C<sub>82</sub>(OH)<sub>-21</sub>O<sub>-7</sub> (2#) (e).

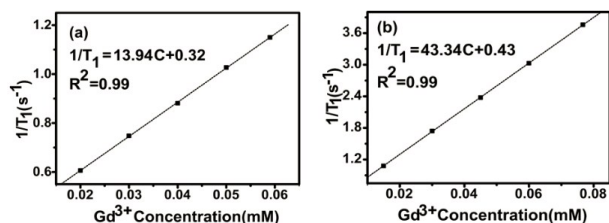


To further substantiate and differentiate the contents of hemiketal structure between Gd@C<sub>82</sub>(OH)<sub>-20</sub>O<sub>-2</sub> and Gd@C<sub>82</sub>(OH)<sub>-21</sub>O<sub>-7</sub>, acid treatment was employed as reported by Chiang et al.<sup>[34]</sup>. Since the fullerenols with hemiketal structure tend to form ketone analogue after acid treatment (from structure 1 to structure 3 as illustrated in Scheme 1), this can be detected readily by infrared spectrum, while those without hemiketal structures keep silent. The FTIR spectra of Gd@C<sub>82</sub>(OH)<sub>-20</sub>O<sub>-2</sub> and Gd@C<sub>82</sub>(OH)<sub>-21</sub>O<sub>-7</sub> are shown in Fig. 1c-1# and Fig. 1c-3#, respectively. Before acid treatment, both of them exhibited characteristic absorption of hydroxylated Gd@C<sub>82</sub>, a broad O-H band at around 3400 cm<sup>-1</sup> and three characteristic bands at 1070, 1390, and 1590 cm<sup>-1</sup>, assignable to  $\nu$ C-O,  $\delta$ sC-O-H, and  $\nu$ C=C, respectively. Moreover, the infrared absorption of Gd@C<sub>82</sub>(OH)<sub>-20</sub>O<sub>-2</sub> displayed a slight blue shift compared with that of Gd@C<sub>82</sub>(OH)<sub>-21</sub>O<sub>-7</sub>, which may ascribe to their different aggregation state induced by formation of hydrogen bond<sup>[35-36]</sup>. However, as shown in Fig. 1c-4#, new absorption was observed for Gd@C<sub>82</sub>(OH)<sub>-21</sub>O<sub>-7</sub> after treating with HCl, while that of Gd@C<sub>82</sub>(OH)<sub>-20</sub>O<sub>-2</sub>

exhibited negligible change under the same condition. This indirectly proved that  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  possessed negligible hemiketal structures compared with that of  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$ .

UV-vis spectra of  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  and  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$  were measured in water. As can be seen in Fig. 1d,  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  exhibited stronger UV absorption than  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$  at the same concentration (determined by ICP), especially in near ultraviolet area. This may ascribe to the structure difference (different contents of hemiketal structures). Besides, the different aggregation behaviors between  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  and  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$ , as revealed by DLS in Fig. 3a, may also be another reasonable explanation. Suppose that  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  disperses much better in aqueous solution than  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$ , then large aggregation are difficult to form and more  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  particles will be exposed to UV irradiation. As a result, stronger UV absorption can be expected.

The cyclic voltammetry (CV) was utilized to certify the structure differences between  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  and  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$  (Fig. 1e). Two oxidation peaks at -0.368 V and 0.174 V were observed for  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$ , which probably correspond to  $\text{Gd@C}_{82}/\text{Gd@C}_{82}^{+}$  and  $\text{Gd@C}_{82}^{+}/\text{Gd@C}_{82}^{2+}$  respectively according to the literature<sup>[37-39]</sup>. In comparison,  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$  has relatively higher oxidation potentials of -0.436 V and 0.211 V, indicating that  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  is more easily oxidized than  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$ . This may be attributed to the presence of hemiketal structures in  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$ , which is supposed as the existence of pre-oxidation of  $\text{Gd}^{3+}@C_{82}^{3-}$ . Therefore, more hemiketals make  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$  more difficult to be further oxidized. All these experimental results above reveal that  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  and  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$  possess different properties due to the different contents of hemiketals.

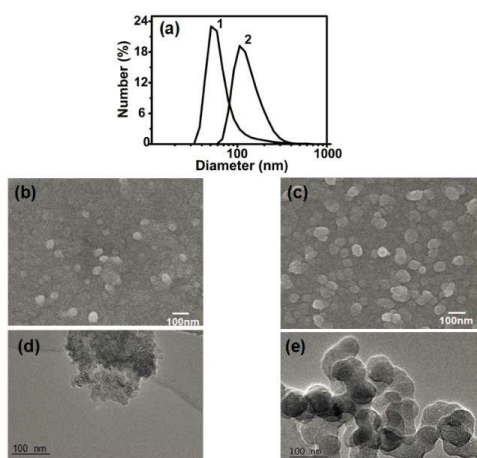


**Fig. 2** Linear relationship between  $T_1$  relaxation rates ( $1/T_1$ ) and  $\text{Gd}^{3+}$  concentrations for  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  (a) and  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$  (b) in water at 0.5 T and 300 K.

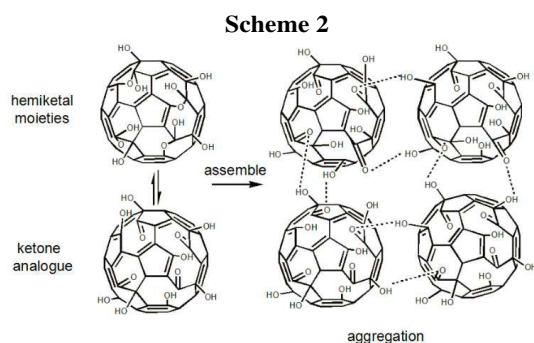
To study the influence of hemiketals on the water proton relaxation, the relaxivities of  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  and  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$  were evaluated at 0.5 T, 37 °C. Surprisingly, as shown in Fig. 2, the measured relaxivity of  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  ( $13.9 \pm 2 \text{ mM}^{-1}\text{s}^{-1}$ ) is significantly lower than that of  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$  ( $43.3 \pm 3 \text{ mM}^{-1}\text{s}^{-1}$ ), which is comparable to that of carboxylic derivatives of gadofullerenes<sup>[20]</sup>. To the best of our knowledge,  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  has the lowest relaxivity among hydroxyl functionalized gadofullerenes so far, since the relaxivities of most  $\text{Gd@C}_{82}$  hydroxyl derivatives investigated to date are located at 40~65  $\text{mM}^{-1}\text{s}^{-1}$  at 0.5 T<sup>[13,26-27]</sup>. The measured relaxivities at 7 T were evaluated as 8.4  $\text{mM}^{-1}\text{s}^{-1}$  for  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  and 21.5  $\text{mM}^{-1}\text{s}^{-1}$  for  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$ , a comparable rule was obtained. (Figure S1-S2 in SI).

Currently, various surface-modifications have been developed for gadofullerene<sup>[40-42]</sup>. Notably, gadofullerenes with hydroxyl groups are more effective to achieve higher

relaxivities than that of other derivatives. Whereas, the prepared  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  by hydrolysis of polycyclo sulfated precursors in this work showed abnormally lower relaxivity. As described in the introduction, the number of hydroxyl groups and aggregating size should play predominant roles in relaxivity of polyhydroxylated metallofullerenes<sup>[14,20-21]</sup>. Although the difference of hydroxyl number may lead to the diversity of relaxivities, the hydroxyl numbers of  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  and  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$  are comparative, which will not induce much relaxivity distinction. Figure S5-S6 in the Supporting Information (SI) shows the relaxivities of  $\text{Gd@C}_{82}(\text{OH})_{-15}\text{O}_{-6}$  and  $\text{Gd@C}_{82}(\text{OH})_{-18}\text{O}_{-8}$  prepared by the alkaline reaction (the structure were estimated by XPS in Figure S3-S4 in SI). Although they possess different hydroxyl numbers, they displayed consistently high and proportionable relaxivities. The measured hydrodynamic diameter of  $\text{Gd@C}_{82}(\text{OH})_{-15}\text{O}_{-6}$  and  $\text{Gd@C}_{82}(\text{OH})_{-18}\text{O}_{-8}$  are both above 100 nm (Figure S7 in SI), suggesting that  $\text{Gd@C}_{82}(\text{OH})_x\text{O}_y$  with high relaxivity is generally accompanied with large aggregation size. It is, then, reasonable to predict that the abnormal relaxivity difference between  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  and  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$  should ascribe to their different aggregation behaviors in aqueous solution. As expected, the hydrodynamic diameter of  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  ( $50 \pm 8 \text{ nm}$ ) is indeed much smaller than that of  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$  ( $105 \pm 10 \text{ nm}$ ) as shown in Fig. 3a. Accordingly, similar results were obtained by both the SEM (Fig. 3b, 3c) and TEM (Fig. 3d, 3e). Additionally, the measured Zeta potential distribution for  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$  was not narrow as that of  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  (Figure S8-S9 in SI), indicating the existence of more complicated aggregation behavior in aqueous solution. All these results reasonably supported the different aggregation behavior between  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  and  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$ . So, the presence of different quantities of hemiketal structure should play an important role in the aggregation of polyhydroxylated gadofullerenes. A possible mechanism is proposed as depicted in Scheme 2. Since the hemiketal structures are prone to convert to ketone analogue, the corresponding carbonyl structure with strong electronegativity is available, which favors the interaction with the adjacent hydroxyl and finally contributes to the formation of hydrogen-bond between gadofullerenols. Comparing with pure hydroxy, it is much easier to form hydrogen bond between hydroxy and carbonyl, and this is verified by the infrared absorption blue shift of  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  compared with that of  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$  as shown in Fig. 1c. This should provide a good explanation for the different aggregation behavior and abnormal relaxivities between  $\text{Gd@C}_{82}(\text{OH})_{-20}\text{O}_{-2}$  and  $\text{Gd@C}_{82}(\text{OH})_{-21}\text{O}_{-7}$ .



**Fig. 3** Size distributions for Gd@C<sub>82</sub>(OH)<sub>-20</sub>O<sub>-2</sub> (1#) and Gd@C<sub>82</sub>(OH)<sub>-21</sub>O<sub>-7</sub> (2#) in water at room temperature (a); SEM micrographs of Gd@C<sub>82</sub>(OH)<sub>-20</sub>O<sub>-2</sub> (b) and Gd@C<sub>82</sub>(OH)<sub>-21</sub>O<sub>-7</sub> (c); TEM images of Gd@C<sub>82</sub>(OH)<sub>-20</sub>O<sub>-2</sub> (d) and Gd@C<sub>82</sub>(OH)<sub>-21</sub>O<sub>-7</sub> (e).



## Conclusions

In this paper, Gd@C<sub>82</sub>(OH)<sub>x</sub>O<sub>y</sub> with different numbers of hemiketal structures were synthesized. Among these compounds, Gd@C<sub>82</sub>(OH)<sub>x</sub>O<sub>y</sub> with more hemiketals showed much higher relaxivities than that with less hemiketals, which could be attributed to their large aggregation in aqueous solution, and this is further confirmed by DLS, SEM and TEM. The investigation shows more hemiketal structures would benefit the formation of intermolecule hydrogen-bond, and finally lead to higher relaxivities.

Generally, this work provides a new strategy to improve the relaxivities of gadofullerenes by introducing more acceptable hemiketal structures to the carbon cage, which could be an effective way of optimizing gadofullerenes based MRI contrast agents.

## Acknowledgements

This work is supported by the National Natural Science Foundation of China (No. 51372251, 11179006), the Key Research Program of the Chinese Academy of Sciences (Grant No. KGZD-EW-T02) and Chinese Academy of Sciences (Grant No. XDA09030302).

## Notes and references

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Electronic Supplementary Information (ESI) available: Supplementary data associated with this article can be found in DOI: 10.1039/b000000x/

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