Highly hydrated paramagnetic amorphous calcium carbonate nanoclusters as an MRI contrast agent

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Amorphous calcium carbonate (ACC), which exists widely in nature, plays a key role as transient precursor in the early stages of biomineral formation1–4. Using a bioinspired strategy, diverse materials with controlled phase, physical and chemical properties can be achieved5,6. The highly hydrated content is a distinctive feature of ACC, and plays a pivotal role in its stabilization6–12. As a metastable phase of calcium carbonate, ACC is unstable in aqueous solution and will transform into crystalline phases rapidly owing to dehydration, ion binding and other factors6–8,13. Therefore, the potential application of highly hydrated ACC is largely ignored, and there are few successful examples to utilize ACC in biomedicine.

One significant parameter to enhance the contrasting performance of gadolinium-based $T_1$ MRI contrast agents is their hydration14. With seven non-paired electrons, the gadolinium ion possesses a large magnetic moment and long electron spin relaxation time, leading to numerous clinically available extracellular gadolinium-based contrast agents for $T_1$ MRI14,15. In virtue of versatile functionalization to interact with biomolecules in vivo and lower gadolinium ion leakage rates benefitting from an inorganic nanostructure, Gd-based inorganic nanoagents attracted considerable attention15,16. Unfortunately, the hydration of gadolinium-based nanoparticles suffers from high-temperature synthesis, though the consequent ion leakage is
minimized by the confined nanostructure compared with chelate complexes.

Herein, we introduce gadolinium ions into the ACC mineralization process, which have been proven to be integrated into the final amorphous calcium carbonate phase. In this amorphous system, gadolinium ions in conjunction with poly(acrylic acid) facilitate enhanced hydration water and stability of nanoclusters, which confine the formation of gadolinium ions by carbonate improve the bio-compatibility and performance, indicating the resulting product has noteworthy MRI contrast agent properties. Furthermore, a mutual effect between the paramagnetic lanthanide gadolinium ion and amorphous calcium carbonate is discovered, which contributes to the maximized hydrated content in the as-prepared amorphous composite nanoclusters and a high longitudinal relaxation. The final paramagnetic amorphous carbonate nanoclusters (ACNC) possess a high water to Ca ratio (water/Ca = 7.2) compared with the normal ACCs (ratios remained constant at about 0.4–1.9). The longitudinal relaxivity of ACNC (37.93 ± 0.63 mM$^{-1}$s$^{-1}$ under 3.0 T) has also benefited from the high water content, which is ten times higher than that of the commercially available MR contrast agent gadopentetate (Gd-DTPA) and highly resistant to ion leakage so it can serve as a potential MR contrast agent.

**Results**

**Characterization of ACNC**

So far, besides different kinds of organic additives such as biomolecules and polymers, magnesium is the only inorganic cation that has been reported capable of stabilizing ACC$^{-2,7}$. The gadolinium ion, whose ionic radius is closer to that of the calcium ion than that of the magnesium, can be regarded as a smaller calcium ion analog with whose ionic radius is closer to that of the calcium ion than that of the complexes$^{15}$. Possessing a high magnetic moment, the gadolinium ion is able to form a chelate complex with a stable organic acid, a solidification process, which have been proven to be integrated into the final amorphous calcium carbonate phase. In this amorphous system, gadolinium ions in conjunction with poly(acrylic acid) facilitate enhanced hydration water and stability of nanoclusters, which confine the formation of gadolinium ions by carbonate improve the bio-compatibility and performance, indicating the resulting product has noteworthy MRI contrast agent properties.

Moreover, a mutual effect between the paramagnetic lanthanide gadolinium ion and amorphous calcium carbonate is discovered, which contributes to the maximized hydrated content in the as-prepared amorphous composite nanoclusters and a high longitudinal relaxation. The final paramagnetic amorphous carbonate nanoclusters (ACNC) possess a high water to Ca ratio (water/Ca = 7.2) compared with the normal ACCs (ratios remained constant at about 0.4–1.9). The longitudinal relaxivity of ACNC (37.93 ± 0.63 mM$^{-1}$s$^{-1}$ under 3.0 T) has also benefited from the high water content, which is ten times higher than that of the commercially available MR contrast agent gadopentetate (Gd-DTPA) and highly resistant to ion leakage so it can serve as a potential MR contrast agent.

**Contribution of Gd ion in ACC-like environment**

In order to investigate the contribution of each component to the highly hydrated content and ACC-like environment, a series of control samples with varying composition were synthesized as listed in Supplementary Table 3, including a Gd-occluded ACC composite (ACC-Gd), PAA-stabilized ACC (ACC-PAA), amorphous gadolinium carbonate (AGC), PAA-stabilized AGC (AGC-PAA) and two carbonate-free chelates (PAA-Ca and PAA-Ca-Gd). XRD patterns and Raman spectra were performed to identify the amorphous phase (Supplementary Fig. 5a, b$^{13}$). The extended X-ray absorption fine structure (EXAFS) results further confirmed the presence of ACC-like environments (Fig. 2a, b).

In the absence of PAA, ACC-Gd showed a typical morphology for aggregated ACC nanoparticles with a uniform distribution of Gd and Ca (Supplementary Fig. 9). The 13C nuclear magnetic resonance (NMR) result of ACC-Gd showed a characteristic vibration at 168 ppm, which indicated the ACC precursor phase in ACC-Gd (Supplementary Fig. 10). FT-IR spectrum showed a consistency compared with the amorphous calcium carbonate phase, and the $v_2$ vibration shifted to lower value indicated the formation of amorphous gadolinium carbonate (AGC) phase (Supplementary Fig. 11$^{4}$). The oxygen (O 1s) peak of AGC’s XPS result was quite similar to ACC’s curve (Supplementary Fig. 12). The XPS spectrum of AGC-PAA exhibited similar profile to ACCN (Supplementary Fig. 13).

The transformations of ACC-Gd were investigated in different mediums (ethanol, water) to further explore how Gd affected the stability of ACC. The XRD results of samples were collected at different time points (Supplementary Fig. 14). After 6 months, calcite, aragonite, and vaterite were all contained in ACNC dispersed in ethanol. In sharp contrast, ACC-Gd was stable in ethanol over 6 months. The fresh-prepared additive-free ACC crystallized rapidly after dispersion in water, whereas ACC-Gd could maintain amorphous phase for tens of minutes. Overall, Gd should play a reasonable role in retarding the crystallization of ACC. More attractively, there was still a higher water content held in ACC-Gd as compared to pure ACC for a long time. According to the TGA result, the weight loss before 300 °C of ACC-Gd and ACC powder exposed to the air over 6 months was more than 10% and only 0.1%, respectively (Fig. 2c). Note that ACC has completely lost its hydration water under these conditions.

**Highly hydrated content of ACNC**

In both TGA and volumetric Karl Fischer titration measurement results, the weight loss before 300 °C could be assigned to the loss of water, and ACNC exhibited the highest water content of calculative 20 wt% among the products with different composition (Fig. 2d). According to
nanoparticles. More importantly, to compare the ratio of water to Gd enhancement of water binding by PAA and Gd in these amorphous gadolinium ion.

Compared with AGC, the presence of ACC in the Gd-ACC composite also resulted in a two-fold increase of the hydrated content per

shape seen in the electron microscopy images (Supplementary

ACNC via the frictional ratio and Perrin function assuming a spherical

centrifugation (AUC) was applied to determine the hydration water of

experiments is shown. The inset in (e) shows SAED of ACNC. The experiments were

repeated three times independently. f k$_2$-weighted EXAFS and g k$_2$-weighted Fourier transform of the EXAFS for the ACNC and ACC standard. Dotted black lines are their best fits. h SAXS patterns of ACNC dispersed in normal saline solution. Red solid line fit sphere particles. i Distance distribution received by SAXS of ACNC dispersed in normal saline solution. j T$_1$ of ACNC powder under an N$_2$ atmosphere with a heating rate of 10 °C min$^{-1}$. k TG-FTIR spectra of ACNC powder under an N$_2$ atmosphere with a heating rate of 10 °C min$^{-1}$.

previous reports, the water contents per mole of calcium in additive-free ACC and PAA-stabilized ACC were typically located in the range of 0.4–1.58 and 1.33–1.93, respectively.$^{2,23}$ In contrary, both ACC-Gd and ACC-PAA showed a higher water to Ca ratio, and the highest ratio was obtained in ACNC (water/Ca = 7.2) (Fig. 2e), indicating a synergetic enhancement of water binding by PAA and Gd in these amorphous nanoparticles. More importantly, to compare the ratio of water to Gd as listed in Fig. 2f, the hydrated content per mole gadolinium in the presence of both ACC-like environment and PAA in ACNC is more than four times that of PAA-occluded gadolinium carbonate (PAA-AGC). Compared with AGC, the presence of ACC in the Gd-ACC composite also resulted in a two-fold increase of the hydrated content per gadolinium ion.

To investigate the origin of the hydration, analytical ultracentrifugation (AUC) was applied to determine the hydration water of ACNC via the frictional ratio and Perrin function assuming a spherical shape seen in the electron microscopy images (Supplementary Table 4). The samples show sedimentation coefficients in the order of 10$^{-12}$ s, which is typical for prenucleation clusters (PNCs) that have similar sizes.$^{26}$ The ACNCs have a diameter of 1.5 nm, which is even smaller than the one found by SAXS, though still in reasonable agreement. Their molar mass of 2230 g/mol indicates that they consist of ~20 CaCO$_3$ ion pairs including the Gd$^{3+}$ ions. The determined amount of bound hydration water in solution of 8.8 mol H$_2$O per CaCO$_3$ (and 23.0 mol H$_2$O per Gd$^{3+}$), is considerably higher than in common ACCs (Fig. 2e, f and Supplementary Table 5). Notably, the water content of ACNC determined by AUC is in reasonable agreement with the results from TGA, considering that the hydration was determined in wet and dry states, respectively.$^6$

**Stable high relaxivity of ACNC**

Besides the contribution of the dopant of the gadolinium ion to the highly hydrated ACC content, a typical paramagnetic behavior was achieved in the amorphous carbonate nanoclusters as shown in the M–H curve (Fig. 3a). Both the high hydration and confinement of the gadolinium ions, the paramagnetic ACNC was conducive to increasing MRI contrast performance. The T$_1$ relaxation times of ACNC dispersed in normal saline at varying concentrations were measured using a 3.0 Tesla MR scanner. As shown in the T$_1$ map (Fig. 3b), the longitudinal relaxivity (r$_L$) of ACNC reached 37.21 mM$^{-1}$·s$^{-1}$ (Fig. 3c), which was ten times higher than that of commercially used Gd-DTPA (3.19 mM$^{-1}$·s$^{-1}$). Besides the contribution from the macromolecules and nanoparticles, ACNC possessed four times higher longitudinal relaxivity compared to PAA-modified amorphous gadolinium carbonate (AGC-PAA) (7.91 mM$^{-1}$·s$^{-1}$), which was attributed to the high hydration content of ACC-like environments. This high content of water may be primarily attributed to the stronger hydration ability of trivalent Gd$^{3+}$ as compared to divalent Ca$^{2+}$ ions,$^6$ which reasonably enhanced inner-sphere and outer-sphere relaxations of the ACC-like environment occluded ACNC.$^{11}$

Here, we further evaluate the effect of the content of PAA on water content and relaxivity. We denoted the input of PAA for the ACNC product as ‘n’. Using the same synthesis methods, several products with PAA inputs of 2n, 3n/2, 4n/5 (labeled ACNC$_{(2n}$PAA)$^n$, ACNC$_{(2n/2}$PAA)$^n$ and ACNC$_{(2n/5}$PAA)$^n$...
ACNC$_{w/20-PAA}$ were obtained under the same experimental conditions. We found a significant increase in water content of the whole product system with PAA added (Supplementary Fig. 15). However, a ‘roller coaster’ effect was also detected as the molar ratio of water molecules per mole of Gd reached its peak with an input size of 1/n (Fig. 3d). In addition, we measured the relaxivities of these samples. Interestingly, its performance was highly consistent with that of water content per Gd in the way that relaxivity of ACNC reached the highest under the condition of 1/n, yet got reduced when the amount of PAA was 2n and ‘n’/2. Specifically, the relaxivity decreased significantly when the amount of PAA was only 10/n (Fig. 3d, e). In light of the results, it was clear that the molar ratio of water molecules per mole of Gd acted strongly on the performance of the products. These comparisons suggested that reactivity was changing along with water content per Gd as they share a similar pathway of changing.

We further designed and fabricated two more amorphous carbono nanoclusters with varying ratios of Gd added for comparison purpose. In the synthesis of ACNC, mol[GdCl$_3$]:mol[CaCl$_2$] equals to 1.5. We also prepared two samples with initial Gd/Ca feed ratio as 1:10 and 1:2 (denoted as ACNC$_{Gd/Ca=1:10}$ and ACNC$_{Gd/Ca=1:2}$, respectively). ACNC$_{Gd/Ca=1:10}$ and ACNC$_{Gd/Ca=1:2}$ were both amorphous phases, and no crystallization peaks could be found in XRD patterns of the two samples. The molar ratio of water molecules per mole of CaCO$_3$ in ACC, AGC, AGC-PAA, and ACNC according to thermogravimetric (TG) analysis and volumetric Karl Fischer titration measurements (n = 3 independent samples). The data show means ± SD. e Molar ratio of water molecules per mole of CaCO$_3$ in ACC, AGC-PAA, ACC-Gd, AGC, AGC-PAA, and ACNC in comparison with the reported value ranges of additive-free ACC (pink region) and PAA-stabilized ACC (orange region) (n = 3 independent samples). The data show means ± SD. f Molar ratio of water molecules per mole of Gd in AGC, AGC-PAA, ACC-Gd, and ACNC (n = 3 independent samples). The data show means ± SD.

After three tests, the $r_1$ value of ACNC was calculated to be $-37.93 \pm 0.63 \text{mM}^{-1} \cdot \text{s}^{-1}$ (Fig. 3g). In addition, $r_1$ and $r_2$ values of ACNC were measured under different magnetic fields (3.0 T and 0.5 T), and its corresponding $r_2/r_1$ ratio was calculated (Fig. 3h, i). The $r_1$ value of ACNC measured on 3.0 T was as high as 38.19 mM$^{-1} \cdot$ s$^{-1}$, and the corresponding $r_2$ value and $r_2/r_1$ ratio were 72.49 mM$^{-1} \cdot$ s$^{-1}$ and 1.90, respectively. Using a low field MR scanner system (0.5 T), the corresponding $r_1$ and $r_2$ values of ACNC were 66.37 mM$^{-1} \cdot$ s$^{-1}$ and 78.04 mM$^{-1} \cdot$ s$^{-1}$, respectively, and the $r_2/r_1$ ratio was 1.18. Based on the results of AUC measurements, the molar mass of ACNC with a diameter of 1.5 nm was 2230 g/mol. The relaxivity density of ACNC can be eventually calculated with the help of the volume and molecular weight (21.46 mM$^{-1} \cdot$ s$^{-1}$/nm$^3$, 17.01 mM$^{-1} \cdot$ s$^{-1}$/kDa, respectively).

High stability is essential for gadolinium-based contrast agents, as transmetallation will lead to the release of dissociated gadolinium ion with reported toxicity such as nephrogenic systemic fibrosis$^{12,17}$. Laurent and Muller reported the poor kinetic inertness against transmetallation for linear gadolinium-based contrast agents such as commercially used Gd-DTPA$^{38}$. As shown in Fig. 3j, the PAA-Ca/Gd chelate exhibited an even worse inertness than Gd-DTPA after exposure to Zn$^{2+}$ in PBS for 48 h. On the contrary, ACNC demonstrably enhanced the stability against transmetallation, due to the gadolinium confinement in ACC-like environments. Furthermore, we addressed a ligand competition assay in homogeneous aqueous solution containing DTPA and ACNC. There was no observable evidence showing that relaxivity of ACNC was significantly altered, which confirmed that ACNC was not affected by the competition between Gd(III) complex and excessive DTPA-free ligand (Supplementary Fig. 17).

**Stability and biocompatibility of ACNC**

The absorption spectra of Arsenazo III is generally used to detect the leakage of gadolinium ion from Gd-based nanocomposite and gadolinium chelates$^{39,40}$. When the Arsenazo III aqueous solution was mixed with Gd$^{3+}$, pink solution turned blue due to the formation of arsenazo-
Precipitation of free Gd\(^{3+}\) ions was suppressed at near neutral (pH = 6) conditions, which prevented leakage of Gd ions, indicating the good solubility product (Ksp) of phosphate than that of carbonate. Therefore, we speculated that Gd(III) was improved leakage of Gd ions was ascribed to the loss of protection provided by phosphate.

Moreover, ACNC was dispersed in human serum at 1 mmol (Gd)/mL, suggesting a good blood compatibility. MTT assay was used as an evaluation of cytotoxicity by co-culturing human renal tubular epithelial cell and Supplementary Fig. 19), indicating the low dissociation risk for ACNC in the retention period in vivo.

To determine whether ACNC cause hemolysis, ACNC at different concentrations in PBS environment, the improved leakage of Gd ions was ascribed to the loss of protection provided by phosphate. Therefore, we speculated that Gd(III) was difficult to leakage from ACNC and exist as ions under physiological conditions.

Gd\(^{3+}\) complex (Fig. 4a). As shown in Fig. 4b, free gadolinium ion at a low concentration of 1 mg/mL was detectable by Arsenazo III mediated colorimetric analysis (Fig. 4d, e and Supplementary Fig. 19), indicating the low dissociation risk for ACNC in the retention period in vivo. It is well known that pH plays a key role in tissue and cellular homeostasis. Moreover, different cellular compartments present variety of acid-base conditions. We used PBS buffers with a pH of 6.0–6.8 to mimic the weakly acidic condition of different intracellular locations. Although the leakage of Ca ions can be observed, the leakage of Gd ion was scarcely detected with 7 days (Supplementary Fig. 18). We presume that Gd(III) within ACNC will tend to form GdPO\(_4\) with phosphate in the PBS buffer due to the lower thermodynamic solubility product (Ksp) of phosphate than that of carbonate. The precipitation of free Gd\(^{3+}\) ions was suppressed at near neutral (pH = 6) conditions, which prevented leakage of Gd ions, indicating the good biosafety of ACNC under weak acid conditions. Under more acidic environmental pH (pH 4.5–5.5) simulated by acetate buffers, the release of Gd ions slightly increases with the decrease of pH (Supplementary Fig. 18). Compared with the results in PBS environment, the improved leakage of Gd ions was ascribed to the loss of protection provided by phosphate. Therefore, we speculated that Gd(III) was difficult to leakage from ACNC and exist as ions under physiological environment.

Moreover, ACNC was dispersed in human serum at 1 mmol (Gd)/mL. To simulate the elevated phosphate concentrations in serum in patients with end-stage renal disease, the same concentration of ACNC was dispersed in human serum supplemented an additional phosphate concentration of 10 mmol/L for 15 days, followed by dialysis at different time points. No leakage of free gadolinium ions was detected in dialysates using ICP-MS and colorimetric analysis (Fig. 4d, e and Supplementary Fig. 19), indicating the low dissociation risk for ACNC in the retention period in vivo.

To determine whether ACNC cause hemolysis, ACNC at different concentrations in serum were incubated with human blood serum at 37 °C for a hemolysis test. According to the standard, ACNC have no hemolysis even at a high concentration of 0.5 mg (Gd)/mL, suggesting a good blood compatibility (Fig. 4f). MTT assay was used as an evaluation of cytotoxicity by co-culturing human renal tubular epithelial cell...
Recent clinical translation of nanosized biomaterials attracted great attention, promising the development of novel and specific nanomedical tools for diagnosis and therapy.\(^{26,31-33}\) The majority of administered nanomaterials were sequestered by liver and spleen, which thus become the major biological barriers for translating nanomedicines.\(^{26}\) To avoid the potential risk in vivo, renal clearance is the desirable and preferred excretion route for medical agents for minimal catabolism and body exposure. However, compared to small molecular contrast agents, nanosized MR contrast agents, especially the FDA-approved nanosized iron oxide injections suffer from their poor renal excretion as a result of their large size distribution (>20 nm).\(^{34}\) The liver is the primary or secondary target of transplanted injection.\(^{35}\) The enhancement of \(T_1\) weighted MRI performance of ACNC was further confirmed by MR angiography in rat, rabbit, and beagle dog. After the bolus intravenous injection of ACNC at a low dose (3 mg/kg bodyweight), jugular, carotid, aorta, and caudal vena cava can be clearly identified (Fig. 5a–c), and the dosage is ~1/5 of the typically employed dose in clinic. The whole-body angiography images of rat, rabbit, and upper body of beagle dog exhibited better angiographic contrast and more details in comparison with that of the Gd-DTPA control group. Meanwhile, semiquantitative analysis clearly displayed that signal intensities in ACNC groups have a remarkable enhancement in comparison to those in Gd-DTPA control groups (Supplementary Figs. 29, 30). As shown in Fig. 5d, the contrast enhancement of ACNC in vivo was further quantitatively reflected by the mean value of signal-noise ratio (SNR) on rabbit and beagle dog, respectively.\(^{36}\)

**In vivo MR imaging and metabolism of ACNC**

The enhancement of \(T_1\) weighted MRI performance of ACNC was further confirmed by MR angiography in rat, rabbit, and beagle dog. After the bolus intravenous injection of ACNC at a low dose (3 mg/kg bodyweight), jugular, carotid, aorta, and caudal vena cava can be clearly identified (Fig. 5a–c), and the dosage is ~1/5 of the typically employed dose in clinic. The whole-body angiography images of rat, rabbit, and upper body of beagle dog exhibited better angiographic contrast and more details in comparison with that of the Gd-DTPA control group. Meanwhile, semiquantitative analysis clearly displayed that signal intensities in ACNC groups have a remarkable enhancement in comparison to those in Gd-DTPA control groups (Supplementary Figs. 29, 30). As shown in Fig. 5d, the contrast enhancement of ACNC in vivo was further quantitatively reflected by the mean value of signal-noise ratio (SNR) on rabbit and beagle dog, respectively.\(^{36}\)

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Nanoparticles detained in the liver could be eliminated from the liver via hepatobiliary clearance. Besides the conventional elimination from liver (Supplementary Fig. 31), the quick renal clearance of ACC from blood vessels was observed in the MRA images after intravenous injection (Supplementary Fig. 32). Moreover, the bladder of beagle dog was also brightened within 20 min in the T1w-MRI images of the whole body on beagle dog immediately (IM) and 20 s after the bolus injection of (i) Gd-DTPA and (ii) ACC, respectively. (C) and (S) represents coronal plane and sagittal plane respectively. d Quantitative measurement of SNR of ROIs in (i) brachiocephalic trunk artery, (ii) left subclavian artery, (iii) left common carotid artery, (iv) right branch of olfactory artery of beagle dog, and that in (v) descending aorta, (vi) aortic arch, (vii) ascending aorta of rabbit, respectively. The time for quantitative measurement of SNR of ROIs was in the “IM” period (n = 3 independent experiments). The data show means ± SD. P was calculated using two-tailed Student’s t-test (**P < 0.01, ***P < 0.001).

Therefore, the scientists studied indefatigably including diverse fabrication methods and clearing pharmacokinetics in order to investigate the translatable performance of Gd-based inorganic nanoparticles. In consideration of the hazard originated from the release of free Gd ions, a specific aim was to reduce the dose of injected nanoparticles via the enhanced performance.

The hydration level plays a significant role in determining the contrasting performance of a MR contrast agent. Unfortunately, the hydration content of Gd-based nanocrystals is limited by traditional high-temperature synthesis processes. Worth mentioning is that the high moisture content, including interior water and deeply located structural water is the most distinctive feature of ACC. However, the potential application of unstable hydrated ACC is largely ignored. Interestingly, the ionic radius of the gadolinium ion is very close to that of the calcium ion, implying a possible interaction between gadolinium ions and calcium ions that can be exploited by us.

In summary, our study confirms mutual effects between the paramagnetic lanthanide gadolinium ion and amorphous calcium carbonate, which is beneficial to maximizing the water content in the obtained amorphous composite nanoclusters. The material is synthesized through a facile one-pot process at room temperature, enabling the large-scale and cost-effective production. Importantly, this high water content contributes to the transparent MRI contrasting enhancement of gadolinium-based nanoclusters. In combination with their low toxicity, partial renal clearance and easy potential for mass production, our work enables further identification of the biomedical potential of ACC composites, and we anticipate that it could lead to a next generation of more efficient diagnostic agents on the basis of amorphous nanoclusters in the future.

Discussion
A number of gadolinium-based contrast agents have been designed and approved worldwide for T1 MR imaging. As a result of a high density of free electrons in the valence band and versatile functionalization to interact with biomolecules in vivo, amorphous nanomaterials serving as contrast agents in various imaging modalities are anticipated to achieve the clinical translation of nanomedicine.

\[ \text{SNR} = \frac{I_{\text{ROI}}}{\text{noise}} \]

\[ \text{P} = \frac{\text{observed frequency}}{\text{expected frequency}} \]

\[ \text{SNR} = \text{signal} / \text{noise} \]

\[ \text{P} = \text{probability of the null hypothesis} \]

\[ \text{P} = \text{probability of the alternative hypothesis} \]

\[ \text{SNR} = \frac{\text{signal intensity}}{\text{noise intensity}} \]

\[ \text{P} = \text{probability of the test statistic} \]

\[ \text{SNR} = \text{signal} / \text{noise} \]
**Methods**

**Materials**

Anhydrous calcium chloride, anhydrous sodium carbonate, and ethyl alcohol were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai). Gadolinium chloride hexahydrate (GdCl$_3$·6H$_2$O, 99.95%) were purchased from Yutai QingDa Fine Chemical Co., Ltd. (Shandong, China). Poly(acrylic acid) (PAA, Mw $\approx 1800$) was purchased from Aldrich. All reagents were used as received without further purification. The commercially used Gd-DTPA was produced by Guangdong Consun Pharmaceutical Group, China (specification: 20 mL).

**Sample preparation**

In a typical procedure to synthesize additive-free amorphous calcium carbonate (ACC), 10 mL Na$_2$CO$_3$ aqueous solution (0.1 mol·L$^{-1}$, 10 mL) was poured into calcium chloride (0.1 mol·L$^{-1}$, 10 mL) aqueous solutions under vigorous magnetic stirring for 15 s. The aqueous suspension after adding 20 mL ethanol were centrifuged at 850 × g for 2 min immediately, then the precipitates were washed by ethanol, centrifuged for 5 min at 5000 × g twice.

In a typical procedure to synthesize ACNC, anhydrous calcium chloride (CaCl$_2$, 0.1 mol·L$^{-1}$), gadolinium chloride hexahydrate (GdCl$_3$, 0.02 mol·L$^{-1}$), and PAA (0.45 g) were dissolved in 10 mL DIW with magnetic stirring. Anhydrous sodium carbonate (Na$_2$CO$_3$, 0.1 mol·L$^{-1}$, 10 mL) was poured into the above solution with vigorous magnetic stirring for 1 min. 20 mL of ethanol was added to terminate the reaction. The mixture was immediately centrifuged for 2 min at 850 × g, and precipitates were re-suspended by DIW, centrifuged for 5 min at 5000 × g twice. For liter-scale synthesis, the reaction volume was scaled up 50 times and proceeded under robust mechanical agitation.

For the synthesis of ACC stabilized by PAA (ACC-PAA), 0.45 g PAA was used in the synthesis process without the addition of gadolinium salt, and the product was washed by ethanol. For the preparation of ACC-Gd, PAA was absent in the synthesis process and anhydrous CaCl$_2$ (0.1 mol·L$^{-1}$) and GdCl$_3$ (0.02 mol·L$^{-1}$) were mixed in 10 mL DIW. Then the obtained ACC-Gd was washed by ethanol.

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**Fig. 6 | Metabolism of ACNC.** a, b The time-dependent distribution of ACNC in plasma of a mice and b beagle dogs within 24 h (n = 5 biologically independent animals). The data show means ± SD. c TEM, d EDS results, and e HAADF-STEM image and EDS mapping of ACNC observed in rat urine collected 12 h post-injection. A representative image of three individual experiments is shown. Inset of e is relative SAED pattern. The experiments were repeated three times independently.
In a typical procedure to synthesize amorphous gadolinium carbonate (AGC) and AGC-PAA, Na₂CO₃ (0.1 mol·L⁻¹, 10 mL) was poured into 10 mL GdCl₃ aqueous solution (0.067 mol·L⁻¹, mol[GdCl₃]·mol[Na₂CO₃] = 2:3) under vigorous magnetic stirring for 15 s, then 20 mL ethanol was poured into the aqueous suspension. After centrifuged at 850 × g for 2 min immediately, the precipitates were washed by ethanol, centrifuged for 5 min at 5000 × g twice. In addition, in the presence of 0.45 g PAA in the GdCl₃ aqueous solution, AGC-PAA was prepared in the same procedure.

Characterization
Transmission electron microscopy (TEM) were carried out on H7700 (Hitachi, Japan) with an acceleration voltage of 100 kV. Cryo-transmission electron microscopy (cryo-TEM) was performed on Tecnai F20 Transmission Cryo-Electron Microscopy. High-angle annular dark field scanning TEM (HAADF-STEM), energy dispersive spectrometer (EDS), and EDS mapping were performed on a field emission high-resolution transmission electron microscope (FEI, Talos F200X). X-ray powder diffraction (XRD) patterns were performed on a rotating anode X-ray diffractometer (SmartLab™ 9 kW). X-ray photoelectron spectroscopy (XPS) was performed on a photoelectron spectrometer (ESCALAB, 250XI, Thermo Fisher, USA). ¹³C nuclear magnetic resonance (¹³C NMR) was performed on 400 MHz Bruker Solid-State Nuclear Magnetic Resonance Spectrometer (Bruker AVANCE III 400 WB). FT-IR spectra were recorded with a Thermo Nicolet 8700 FT-IR spectrometer at room temperature. Raman spectra were recorded using a Horiba LabRAM high-resolution (HR) Evolution Raman spectrometer. The light source of the microscope was transferred to diode laser (780 nm). The spectra were scanned for 3 × 10⁶ s⁻¹. Thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), and thermogravimetry analysis coupled with Fourier transform infrared spectroscopy (TG-FTIR) were recorded at a heating rate of 10 K min⁻¹ under nitrogen flow on a TGA thermal analyzer (NETZSC STA 449F3). Magnetic investigation was carried on a superconducting quantum interface device (SQUID) magnetometer (Quantum Design SQUID-VSM). ICP-AES were performed on an Optima 7300 DV instrument. UV-vis spectra were measured on a Shimadzu UV-2600 spectrophotometer at room temperature.

Small-angle X-ray scattering (SAXS)
Small-angle X-ray scattering (SAXS) analysis were performed on a SAXSpoint 2.0 (Anton Paar). The size distribution function as determined by SAXS was evaluated using GIFT (Generalized indirect Fourier transformation) software with pre-condition of homogeneous spheres weighted by number. It is calculated using the following formula:

\[ I(q) = \int_0^{R_{\text{max}}} R^2 \cdot D_N(R) \left( 3 \sin(qR) - qR \cos(qR) \right)^2 \, dq \]

EXAFS sample preparation, measurement, and data fitting
X-ray absorption spectroscopy (XAS) measurements at the calcium K-edge (4.0381 keV) were undertaken at the Elettra synchrotron Italy, operating at an energy of 2 GeV and current of 300 mA. Samples were ground in an agate mortar, diluted with graphite powder, and compacted into thin pellets. An optional sample thickness of ~300 µm and optimal calcium concentration after optimal dilution with graphite powder was prepared for each sample including ACNC, ACC-Gd, and ACC standards. Three full scans per sample of the X-ray absorption near edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) were collected in transmission mode using a FMB-OXFORD ion chamber detector, in steps of 5 eV in the pre-edge region (from 3738.43 to 4028.53 eV) and 0.2 eV in the edge region (from 4061.51 to 4061.71 eV), gradually increasing to 2.6 eV in the post-edge region (4061.71 to 4589.30 eV) up to k = 13. Alignment, energy calibration and de-glitching were performed using built-in features of the Athena software package. The first coordination shell around calcium (Ca–O) was fitted by generating a single scattering pathway of Ca–O based on crystallographic data of calcite. Analysis of single scattering pathways was undertaken from 1.3 to 2.53 Å in R space on Fourier transformed k²-weighted data from 3.11 Å⁻¹. Standards errors associated with the EXAFS data fitting over the k-range used here are 15% for the first-shell coordination number, 0.03 Å for the radial distance, and 15% for the Debye-Waller factors.

Independent data points were determined by Stern’s rule defining the fundamental limitation to the amount of information that can be determined by XAFS. According to Stern’s rule, the number of parameters (Npar) used for the fit should be strictly inferior to the number of independent (Nind) parameters defined as ΔkΔR/π² + 2, where Δk and ΔR are the fitted range in k and R space, respectively43,44. If Nind < Npar, the model cannot be taken as a proof of the coordination environment because the data set is underdetermined by the level of complexity of the model. In this case, Nind = 7.5 for ACC standard, ACC-Gd, and ACNC sample, Nind = 8.16 for ACC-PAA and Npar = 4 as such than Nind > Npar. The multi-component model yielding the best fit of the experimental data is considered as reasonable and can thus be considered as the ‘true’ representation to characterize the calcium coordination environment. Linear combination was performed on the XANES and near-EXAFS region (from 30 Å⁻¹ before to 80 Å⁻¹ after edge jump). Samples ACC-Gd, ACNC, and PAA-Gd/Ca were compared to standards samples ACC-PAA and PAA-Ca.

Analytical ultracentrifugation (AUC)
The AUC measurements were performed on a modified Optima XL A (Beckman Coulter, Palo Alto, CA, United States) using an absorbance optics and an advanced Rayleigh interference optics developed by Nanolytics (https://www.nanolytics-instruments.de/interference_optics_alda). 12-mm path length double-sector titanium centerpieces with sapphire windows (Nanolytics, Potsdam, Germany) were used for all experiments. For ACNC sample measurement, the original ACNC sample was pre-sedimented on a UNIVERSAL 320 Hettich centrifuge (Hettich, Tutlingen, Germany) for 20 min at 9 000 rpm, corresponding to a centrifugal force at 6000 × g, 360 µL of pretreated ACNC solution was used in the sample sector and 360 µL of a 1:175 diluted (with 0.154 M NaCl) pre-reaction PAA-Ca/Gd solution (aqueous mixture including PAA, calcium chloride and gadolinium chloride) in the reference sector. For PAA sample measurement as reference, 10 mg PAA (1800 Da) were dissolved in 1 mL of 0.154 M NaCl solution. All samples were investigated at 20 °C and 60 000 rpm, corresponding to a centrifugal force up to 290,000 × g.

Sedimentation velocity data were evaluated with Sedfit and UltraScanIII. In Sedfit (Schuck, P. P., SEDFIT version 16.1c. http://analyticalultracentrifugation.com/download.html), the ls-g*(s) and continuous c(s,ff0) models were used. For calculation of the sedimentation coefficient distributions g(s) with the least-squares-g*(s) model, data were fitted with a Tikhonov-Phillips regularization using a confidence level (F-ratio) of 0.683 and a resolution of 100 grid points. For determination of the sedimentation particle density with the c(s,ff0) analysis, data were fitted with a maximum entropy regularization using a confidence level (F-ratio) of 0.683 and a resolution of 100 grid points in s and 10–20 grid points in f0. The 2D c(s,ff0) distributions were plotted using MATLAB software version R2017b, 64 bit from MathWorks. For calculation of the sample density from 2D distributions, a MATLAB mask script developed by Quy Khac Ong was used (Institute of Materials, École Polytechnique Fédérale de Lausanne (EPFL), Station 12, 1015 Lausanne, Switzerland. E-mail: quy.ong@epfl.ch). For characterization of anisotropy, UltraScanIII (Demeler, B. UltraScan version 4.0, release 2783. A Comprehensive Data Analysis Software Package for Analytical Ultracentrifugation Experiments. The University of Lethbridge, Nature Communications | (2022) 13:5088 | https://doi.org/10.1038/s41467-022-32615-3)
Department of Chemistry and Biochemistry. http://www.ultrascan3.ausolutions.com/download.php was used for performing the two-dimensional spectrum analysis (2DSA)\(^1\). The 2DSA-Monte Carlo (MC) analyses were performed with 50 iterations on a supercomputer.

The function defining the shape, flexibility, and degree of solvation (by water, salt ions, and any other solvent molecules) of the macromolecule is the Perrin translational frictional function, \(P\) (see following equation). This degree of water association is termed the hydration of the solute, \(\delta\), and is defined as the mass in grams of associated solvent per gram of anhydrous solute.\(^5\)

\[
P = \frac{f}{f_0} \left( \frac{v}{v_0} \right)^{1/3} = \frac{f}{f_0} \left( \frac{\bar{v}}{\bar{v}_0 + \bar{v}_H_2O} \right)^{1/3}
\]

where \(f/f_0\) is the frictional ratio (which is the dimensionless ratio of the observed translational frictional coefficient \(f\) to that of an equivalent spherical molecule of the same anhydrous mass and density \(f_0\)), \(\bar{v}\) is the partial specific volume (cm\(^3\)/g) of the solute and \(\bar{v}_c\) is the hydrated specific volume (the volume occupied by the solute and associated solvent per unit mass of the anhydrous solute) and \(\bar{v}_H_2O\) – specific volume of water given as:

\[
\bar{v}_c = \bar{v} + \delta \bar{v}_H_2O
\]

If all excess friction is due to the hydration and the solute is a sphere, then \(P\) is \(3^{1/3}\) and the hydration can be calculated as:

\[
\delta = \left[ \left( \frac{f}{f_0} \right)^{3} - 1 \right] \bar{v}_H_2O = \left( \frac{f}{f_0} \right)^{3} - 1 \bar{v}_H_2O
\]

Hydrated content analysis
The moles of water molecules per mole of CaCO\(_3\) was thus calculated according to the result of inductively coupled plasma atomic emission spectroscopy (ICP-AES), TG analysis, and volumetric Karl Fischer titration measurement. Titration measurement was performed on a Karl Fischer moisture titrator (V105S, volumetric KF titrator, Mettler Toledo). The hydrated content was assigned to be the weight loss starting from the endothermic peak to 300 °C, while the content of Ca\(^{2+}\) and Gd\(^{3+}\) ions were determined by ICP-AES. The molar ratio between water and Ca\(^{2+}\) ions in ACC can thus be calculated according to previous reports\(^6\).\(^7\).

Leakage studies of Gd\(^{3+}\) ion
The detection of leakage of gadolinium ion was carried out using a method reported previously\(^8\).\(^9\). ACNC were dispersed with a simulative physiological environment including normal saline, human serum, and human serum supplemented an additional phosphate solution at each time interval for analysis and then replaced with fresh 50 mL PBS or acetate buffers with different pH and incubated with shaking at 50 rpm at 37 °C. We collected all the surrounding PBS or acetate solutions at each time interval for analysis and then replaced with fresh 50 mL PBS or acetate buffers. In order to prevent possible interference which the free metal ions generated from degradation precipitate with the phosphate ions in the PBS buffer, 1 mL of freshly prepared chloroacetic acid (HNO\(_3\)/HCl = 3:1) was added to the collected surrounding PBS or acetate solution, leading to a strong acid environment (pH < 3.0) of the mixed solution. Ultimately, the concentrations of Gd ion in surrounding solutions were determined by ICP-AES.

Degradation experiments
1 mL ACNC aqueous solution (5 mM Gd) was added into 1 mL DTPA solution (5 mM) and the homogeneous solution was taken for measurement. Compared with the commercial Gd-DTPA, the concentration of Gd-DTPA solutions was identical to ACNC solutions as 5 mM Gd. The measurements and calculations were consistent with transmetallation study.

Transmetallation study
ACNC, Gd-DTPA, and PAA-Ca/Gd samples were freshly prepared. At \(t = 0\), each sample (2.5 mM Gd) and ZnCl\(_2\) aqueous solution (250 mM, 20 μL) were mixed in a 2 mL phosphate buffer. Then, 1 mL mixed solution was contained in a chromatographic bottle for measurement.

The measurements were carried out at 37 °C under a 0.5 T NMI20- Analyst NMR Analyzing and Imaging system. TR/TE = 100/5.6 ms. Longitudinal relaxation times were measured at different points in time.\(^10\) The relaxation rate at \(t = 0\) (denoted as \(R_1(0)\)) was calculated by the formula \(R_1 = (1/T_1)\). The relaxation rates at other time points were all respectively calculated and recorded as corresponding \(R_1(t)\), for the purpose of assessing the 2-day transmetallation through monitoring the ratio of \(R_1(0)/R_1(0)\).

Ligand competition assay
1 mL ACNC aqueous solution (1 mg (Gd)/mL) was introduced into dialysis bags (MWCO: 1000 KD). The bags were then placed in 50 mL PBS or acetate buffers with different pH and incubated with shaking at 50 rpm at 37 °C. We collected all the surrounding PBS or acetate solutions at each time interval for analysis and then replaced with fresh 50 mL PBS or acetate buffers. In order to prevent possible interference which the free metal ions generated from degradation precipitate with the phosphate ions in the PBS buffer, 1 mL of freshly prepared chloroacetic acid (HNO\(_3\)/HCl = 3:1) was added to the collected surrounding PBS or acetate solution, leading to a strong acid environment (pH < 3.0) of the mixed solution. Ultimately, the concentrations of Gd ion in surrounding solutions were determined by ICP-AES.

In vitro biocompatibility studies
Human renal tubular epithelial (HK-2) cell and human immortal keratinocyte (HaCaT) cell line were cultured in Dulbecco’s modified Eagle’s medium. Cell viability was performed by standard MTT method. Briefly, cells were plated at the density of about 1 × 10\(^4\) cells per well in 96-well plate, and incubated for 12 h. Then DMEM culture medium with ACNC were added, and cells were exposed to ACNC at various concentrations for 24 h and 48 h. Then 10 μL of MTT solution (5 mg/mL in PBS) was added to each well for additional 4 h incubation at 37 °C. After removing the medium, 150 μL of DMSO was added to dissolve the formed formazan crystals in each well, and the optical density of the solution was measured at 570 nm using Microplate Reader (BioTek Instruments, USA). HK-2 cell was used for mitochondrial membrane potentials (MMP) assay, TdT-mediated dUTP Nick-End Labeling (TUNEL) assay, and 5-Ethynyl-2'-deoxyuridine (EdU) assay. Mitochondrial membrane potential assay kit with J-aggregates (JC-1) (Beyotime, Shanghai, China), one-step TUNEL apoptosis assay kit (Beyotime, Shanghai, China), and EdU cell proliferation kit with Alexa Fluor 555 (Beyotime, Shanghai, China) were employed.

Blood studies
The blood for the experiment was professionally collected from volunteers by medical professionals in blood transfusion department of the first affiliated hospital of Anhui medical university. The blood studies were carried out in accordance with the protocols approved by the Ethics Committee of University of Science and Technology of China and Anhui Medical University (license number: 20170267, 20170268).

Animal studies
Specific pathogen Free (SPF) BALB/c mice (male, 6 weeks), New Zealand rabbit (male, 2 kg), and Beagle dog (male, 6 kg) were purchased from Anhui Medical University. Animal studies were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The
in vivo compatibility studies and in vivo MRI studies were under protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Anhui Medical University (LLSC20170299, LLSC20170300) and The First Affiliated Hospital of University of Science and Technology of China (2021-N(A)-041).

**In vivo biocompatibility studies**

ACNC normal saline solutions were intravenously injected in mice (via tail vein) at a dosage of 5 mg Gd/kg bodyweight and Beagle dog (via hindlimb vein) at a dosage of 9 mg Gd/kg bodyweight. For evaluation of blood index and biochemical index, mice were sacrificed by anesthesia for 3 days and 3 weeks after intravenous injection, respectively. Blood of Beagle dogs were collected every two weeks via hindlimb vein. Blood samples were collected by the anticoagulant blood collection tubes and separating gel tubes. The examination of blood index and biochemical index were carried out on Sysmex XE2100 and Vitros 5600, respectively.

**In vivo metabolic study**

ACNC normal saline solutions were intravenously injected into twenty mice (male, 20 g) at 3 mg Gd/kg bodyweight (0.5 mg Gd/mL, 120 μl) via rapid manual injection for a simulated bolus injection study. These mice were randomly divided into four groups, and various organs were resected from the mice in each group 1, 7, 15, and 30 days after intravenous injection, respectively. The urine samples were collected at 12 h after i.v. injection of ACNC on rat at 5 mg Gd/kg bodyweight, followed by dialysis at 25°C. After 24 h, the dialysates in dialysis bags (MWCO ~ 1000 Da) were collected for further characterization.

**MRI acquisition and analysis**

ACNC and Gd-DTPA dispersed in normal saline at gradient concentrations in 5 mL microtubes were placed on a support immersed in NISO₄ solution. The concentrations of gadolinium were measured by ICP-AES. MR T₁ map was acquired using an inversion recovery sequence on a 3.0 Tesla MR scanner (Trio Tim, Siemens) equipped with a local head and neck coil, TR = 3.6 ms, TE = 1.65 ms, FOV: 240 × 320 mm². The time of acquisition (TA) was 24.62 s. Flip angle (FA) was 20. Acquisition matrix was 288 x 512. Acquisition number and number of averages were both 1. The detailed parameters of MR angiography of beagle dog were as follows: A local head and neck coil, spine coil, and radiofrequency body transmit coil were used. TR = 3.19 ms, TE = 1.28 ms, FOV: 240 × 320 mm². The time of acquisition (TA) was 20.74 s. Flip angle (FA) was 16. Acquisition matrix was 288 x 512. Acquisition number and number of averages were both 1. The images were processed firstly by Siemens Syngo MR workstation.

Signal-to-noise ratio (SNR) was calculated in a single image (k) based on two separate regions of interest (ROIs). One in the vessel of interest (ROIvessel) were measured by placing a signal at the same ROI in the same slice both in Gd-DTPA and ACNC group, which was recorded as the mean signal intensities of vessel (Svessel). One in the image background (ROImbackground) was located in an artifact-free homogeneous area, which was defined as the background signal (Sbackground). SNR corresponds to the ratio between Svessel and Sbackground was calculated using Eq. (5):

\[
\text{SNR}_{\text{vessel}}(k) = \frac{S_{\text{vessel}}}{S_{\text{background}}} = \frac{\text{mean}_{k}(S_{V}(r,k))}{\sqrt{\frac{1}{n} \text{stddev}_{k}(S_{V}(r,k))}} = \frac{m_{\text{vessel}}}{\sqrt{n - 1}}
\]

Three ROIs were selected in descending aorta, aortic arch, and ascending aorta on rabbit, and four regions in brachiocephalic trunk artery, left subclavian artery, left common carotid artery and right branch of ophthalmic artery were measured on beagle dog, respectively.

**Statistical analysis**

All data were expressed as mean ± SD. Two-tailed Student’s t-test was used for statistical analysis of between two groups comparison, and one-way ANOVA was used for statistical analysis of among multiple groups comparison. A value of *P < 0.05* was considered statistically difference (*P < 0.05, **P < 0.01, ***P < 0.001).* Statistical significance was determined using SPSS statistics 17.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The authors declare that the data generated in this study are provided in the paper and Supplementary Information, or available from the corresponding authors on request.

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S.H.Y. and Y.L. conceived the idea, designed the experiments, and supervised the research. L.D., C.S., Y.L., Y.Z., L.B.M., D.G., H.C., R.R., Y.D.W., and F.L. carried out the synthetic experiment and analysis. D.G., J.A., H.C., and L.D. worked on the EXAFS measurement and analysis. L.D., Y.Z., H.L.G., Z.P., H.Q.W., X.Y., and F.L. processed the biocompatible evaluation. L.D., Y.J.X., Y.D.W., Y.L., H.L.G., Z.P., X.Y., F.L., and C.S. worked on the animal experiments. L.D., Y.J.X., C.S., D.G., Y.L., H.C., and S.H.Y. wrote the paper, all authors discussed the results and commented on the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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