

Carbon coated microspheres containing nanosized Gd(III) oxidic phases for multiple bio-medical applications†

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Novel sub-microsized graphitic carbon shells embedding nanometric Gd(III) oxidic phases feature thermal and chemical inertness with enhanced T_2 relaxation in aqueous dispersions, thus representing potential candidates for dual diagnostic (magnetic resonance imaging) and therapeutic (neutron capture therapy) applications.

The outstanding results achieved in many fields of bio-medicine have stimulated chemists to design and develop new micro/nano-sized particles endowed with peculiar characteristics.¹ For an efficient visualisation and/or treatment of cell-targeting, cell-tracking and cell-therapy, the use of micro- or nano-sized carriers is often required. Furthermore, only these systems are likely to offer a viable solution for the emerging need for multi-modal agents that simultaneously tackle diagnosis and therapy.² Being in tight association with or even entrapped by cells, the particles must be stable enough to avoid the release of any toxic substance during their residence lifetime in living organisms. The inertness toward biological molecules is therefore a primary requisite. Typically, for d- or f-block metal ions this is achieved by robust ligand chelation or irreversible encapsulation inside bio-compatible hosting matrices.³

Herein we report our preliminary results on the synthesis, characterisation and applicative evaluation of a material based on gadolinium(III) that may find potential simultaneous applications as a diagnostic tool in magnetic resonance imaging (MRI) and X-ray techniques as well as as a therapeutic tool in neutron capture therapy (NCT).⁴

Recently, the incorporation of Gd(III) ions within nanostructured carbon hosts (*i.e.*, shortened single-walled oxidised carbon nanotubes) has provided very interesting results for potential MRI applications,⁵ opening the way for the use of

different carbon matrices for paramagnetic ion delivery. However, a serious limitation is represented by a generally labile inclusion of the metal ions or clusters within the carbonaceous host. Moving from all these perspectives, we have designed and synthesised a system in which Gd(III) ions are irreversibly included inside graphitic sp^2 outer carbon shell coatings (GdOx@C). The composite GdOx@C material can be simply and cheaply prepared *via* precipitation of saturated aqueous solutions of sodium salts of hydrophilic humic acids (HA) in the presence of GdCl₃. HAs are variously oxidised isomeric carbon substrates, which contain in their structures a significant partial extent of graphitic frameworks.⁶ The multiple oxygen groups present⁷ (and, namely, the carboxylic moieties) may promptly complex lanthanide(III) ions, with subsequent massive precipitation from aqueous solution. They were therefore selected as ideal inexpensive commercial starting precursors for the complexation of atomically-isolated Gd(III) ions. Typically, 5 g of HA sodium salts were dissolved in 500 mL of water and precipitated quantitatively with 2.5 g of GdCl₃. A successive pyrolytic thermal treatment up to 1000 °C in a tubular oven, under inert N₂ conditions, yields the final product (see further experimental details reported in the ESI, section 1†).

The excess of gadolinium external to carbon shells was removed by a two-step procedure. Firstly, the crude material was suspended and stirred for 2 h in an aqueous solution of HCl at pH < 1 where the outer gadolinium is solubilised as the Gd(III) hydrated cation. After decantation, the supernatant solution was removed. In the second step, the precipitate was re-suspended and stirred for 2 h in a concentrated DTPA solution at pH 7, in order to ensure the complete removal of all the residual extra-capsular metal traces, as checked by relaxometric measurements.

In order to increase the hydrophilicity of the carbonaceous shells and favour water-suspendability of GdOx@C, pendant zwitterionic diazonium arene sulfonic groups were covalently reacted on the external carbon layers of the capsules in a 3 : 2 THF : H₂O mixture. The isolated sulfonated product (hereafter GdOx@C-ArSO₃⁻, Fig. 1) was re-dispersed in water and the pH was readjusted to *ca.* 7 with NaOH. In this way, the effective water-suspendability is much improved (up to *ca.* 1.5 mg mL⁻¹). Fresh solutions of GdOx@C-ArSO₃⁻ are visibly stable for several hours, albeit the actual suspendability of the different shells tends to vary, according to the capsule size distribution (ESI sections 1 and 3†). Multiple independent syntheses ensured the substantial reproducibility of the obtained materials.

GdOx@C-ArSO₃⁻ has been characterised by means of multiple FT-IR and Raman spectroscopies, thermo-gravimetric

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† Electronic supplementary information (ESI) available: Full details on the synthesis of the GdOx@C-SO₃⁻ material and the XRPD, SEM, TEM, TGA, Raman and FT-IR characterisation are provided. See DOI: 10.1039/b811554k

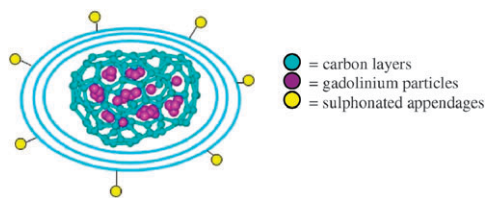


Fig. 1 A model representation of the $\text{GdOx}@C\text{-ArSO}_3^-$ material.

analysis (TGA), X-ray powder diffraction (XRPD), scanning electron microscopy–energy dispersive atomic X-ray fluorescence (SEM-EDAX) and transmission electron microscopy (TEM). The capsular materials feature enhanced thermal and chemical stability and inertness, due to the protective carbon coating provided by the outer graphitic layers.

Raman spectroscopy, both in the solid state and in solution, unambiguously displays the vibrational activity of the two diagnostic G and D bands (at *ca.* 1600 cm^{-1} and 1280 cm^{-1} , respectively),⁸ for the sp^2 graphitic carbon of the outer coating of $\text{GdOx}@C\text{-ArSO}_3^-$.

The actual content of the inorganic component embedded within the $\text{GdOx}@C\text{-ArSO}_3^-$ material was evaluated after an aerobic TGA degradation (25–1000 °C) pathway. Approximately 45 wt% of $\text{GdOx}@C\text{-ArSO}_3^-$ material is composed of a residual inorganic phase, persisting over 500 °C. The remaining combustible fraction was assigned to carbon of the outer coating. It is noteworthy that the comprehensive air stability of the capsular matter is maintained up to *ca.* 400 °C, revealing a remarkable structural stability that efficiently preserves the inner gadolinium content from chemical interaction with the outer solvent media.

SEM-EDAX microscopy atomic fluorescence analyses indicated Gd/C and S/C atomic % values within the 0.38–0.84 and 1.46–2.13 ranges, respectively. Fig. 2(a) shows a typical SEM image of the capsules. They are grossly spheroidal objects, in the micrometre scale on average, with relative predominance in the 0.8–0.9 μm range, a few structures being actually smaller and nano-sized (ESI section 3†).

TEM microscopy allows further investigation of the intimate composition of the capsular materials. In Fig. 2(b), a typical TEM section image shows the nanometric composition of the multiple irregular particles of the inner inorganic phase (darker regions), while the outer protective coating (thin lines) is revealed to be composed of a few parallel graphitic carbon layers, typically 3–7, asymmetrically grown around the paramagnetic metal centres. The XRPD profiles indicate the presence of amorphous graphitic carbon layers⁷ and, as expected

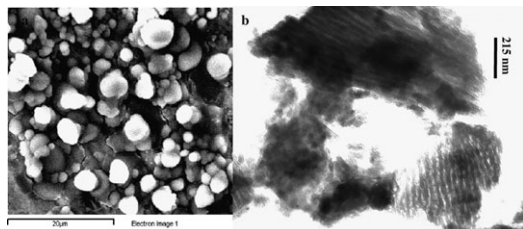


Fig. 2 SEM (a) and TEM (b) images of the $\text{GdOx}@C\text{-ArSO}_3^-$ material.

from the high-temperature pyrolytic conditions, of inorganic gadolinium oxidic phases (ESI section 2†).⁹

The presence of inner oxo-phases of paramagnetic Gd(III) provides the capsules with the ability to reduce the water proton relaxation times T_1 and T_2 . Therefore, they represent interesting candidates for a potential employment in MRI applications.¹⁰ Longitudinal and transverse relaxation rates ($1/T_1$ and $1/T_2$) were measured at variable field strengths using a classic multiblock inversion recovery sequence for T_1 , and Carr–Purcell–Meiboom–Gills (CPMG) spin echo sequence with a refocusing pulse for T_2 , as well as measuring the half band line width for T_2^* . In particular, high relaxivities can be achieved for negative contrast ($1/T_2$) by exploiting the reduction of the phase coherence of water protons diffusing near the particle surface. Notably, the irregular spherical shape and the asymmetric distribution of the inner oxidic phase in $\text{GdOx}@C\text{-ArSO}_3^-$ materials may represent a further favourable contribution. The gain in relaxivity is then dependent on the extent of particle magnetisation and the frequency of the applied field. For determination of the relaxivity (r_{ip} , $i = 1, 2$, *i.e.*, the increase in the water proton relaxation rates normalised to 1 mM concentration of Gd(III)), it is imperative to accurately determine the metal content and to investigate the diamagnetic contribution to r_{ip} . The Gd concentration was assessed by ICP-MS measurements. In this case, mineralisation by HNO_3 digestion was assisted by prolonged sonication under heating at 60 °C, because of the resistance of the outer carbon layers of the capsules. The diamagnetic contribution to r_{2p} was calculated by preparing the equivalent lanthanum, sodium and hollow carbon shells. The suspensions of the three materials gave $R_2 \approx 0.8 \pm 0.2 \text{ s}^{-1}$. The plots of r_{ip} and r_{2p}/r_{1p} are shown in Fig. 3.

The strong dependency of r_2 on the Larmor frequency is clearly evident, whereas r_1 is nearly frequency independent. The quadratic dependency of the r_2/r_1 ratio from the magnetic field strength outlines the potential of these systems as negative MRI contrast agents. This ratio is sensibly higher than that measured recently for related hybrid Gd_2O_3 nanoparticles ($r_2/r_1 \approx 29$ at 7 T).¹¹ Further enhancement of the relaxivity will require an optimized control of the particles' size and/or the thickness of the external carbon coating.

Since the surface of the microspheres is made up of multiple graphitic layers, the magnetic core is well shielded from the bulk solvent molecules and water molecules cannot have direct access to the Gd(III) ions. Therefore, the relaxation

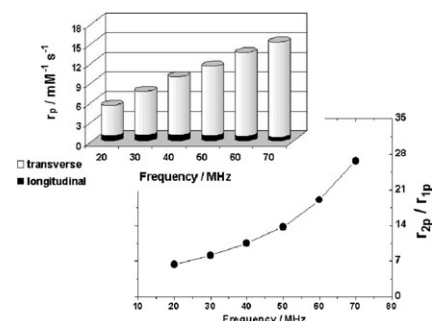


Fig. 3 Top: frequency dependence of r_{ip} ($i = 1, 2$) at 298 K and pH 7. Bottom: r_{2p}/r_{1p} ratio vs. frequency.

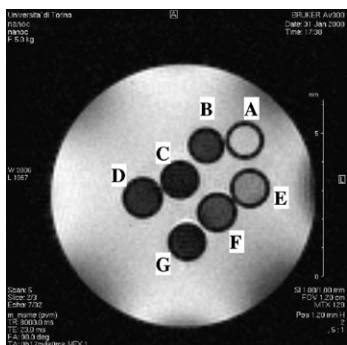


Fig. 4 Cell cultures displaying the actual GdOx@C-ArSO_3^- uptake both in the presence (B, C, D) and in the absence (E, F, G) of polyarginine. The negative contrast provided by the T_2 -specific material with respect to the control cells (A) is apparent.

mechanisms are likely to involve both outer-sphere water molecules and water molecules hydrogen-bonded to the polar solubilising groups grafted on the graphitic surfaces.¹²

As each particle contains *ca.* $2\text{--}5 \times 10^{10}$ Gd atoms, one single particle appears sufficient for the *in vivo* MR visualization of a cell. Such an electron-dense material should be well suitable for X-ray detection. Furthermore, these GdOx@C-ArSO_3^- particles may find application in NCT as Gd-157 (15% natural abundance) shows an excellent ability to capture neutrons (its cross section is 66 times larger than the standard NCT boron-10 nucleus). In fact 10^{10} is just in the right range of Gd atoms that appears to be sufficient for cell disruption in NCT.¹³

NEURO-2A cells were selected for preliminary cellular uptake experiments. They were seeded in Petri dishes (6 cm diameter) at a density of about 3×10^5 cells per cm^2 . After 24 h, cells were ready for the uptake experiments. Cell labeling was performed at 4°C , by addition of different aliquots of GdOx@C-ArSO_3^- , with or without polyarginine additive, to culture cells. The positively charged bio-compatible poly arginine polymer is expected to act as an electrostatic stabilizing linker between the particles and the cellular surface,¹⁴ both negatively charged. After the labeling procedure, a viability test, using trypan-blue as a cell marker, was performed resulting in good viability (75–80%) for cells treated with GdOx@C-ArSO_3^- at different concentrations, both alone and in the presence of polyarginine. After 5 min at 4°C , cells were washed three times with 5 mL ice-cold PBS buffer and were therein recovered from the Petri dishes, with 2 mL EDTA solution, as is usually done for cell detachment. For recording MR images, the cells were collected in 50 μL of PBS and then transferred into glass capillaries that were placed in an agar phantom. The images were acquired by standard T_2 -weighted spin-echo sequence (TR/TE/NEX = 3000/10/6, FOV = 1.2 cm, one slice = 1 mm). The T_2 weighted MR image, recorded on a Bruker300 spectrometer operating at 7.1 T on pellets of cells treated with Gd-loaded carbon capsules, with or without the polyarginine, is reported in Fig. 4. At this magnetic field, r_2^* is $\sim 40 \text{ mM}^{-1} \text{ s}^{-1}$.

The labeled cells appear markedly hypo-intense with respect to the control ones. The observed T_2 values are 13.2 ms for

labeled cells with low concentration of GdOx@C-ArSO_3^- (0.2 mM) and polyarginine, 35 ms for labeled cells with the same concentration of GdOx@C-ArSO_3^- without polyarginine, and 59 ms for unlabeled cells respectively. Clearly there is still some unspecific binding, but these results show that, when the highly negatively charged particles and the polyarginine are simultaneously present, high amounts of Gd effectively accumulate at the cell surfaces.

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