Metal Free Dual Modal Contrast Agents based on Fluorographene Quantum Dots

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Fluorographene quantum dots prepared from fluorinated graphene oxide (FGO) show a linear dependence of the magnetization on the applied field. This is further supported by DFT calculations taking into account a few possible systems of functionalized graphene quantum dots. The inherent magnetism, high concentration of fluorine and cyto-compatibility of these quantum dots promise potential application as a dual modal agent for proton and ¹⁹F based Magnetic Resonance Imaging which is investigated here. A metal free dual modal contrast agent would bring about a great change in the efficiency and resolution of this widely used imaging tool.

Introduction

Carbon and its allotropes have been studied for their unique properties, which offer immense possibilities for future technological applications in energy devices, solar cells and medical applications [1-3]. Magnetism in carbonaceous materials are of immense importance due their unprecedented applications in spintronics, and bio-sensors [4,5]. Graphene, a member of the carbon family, in the absence of any d or f orbital electrons is inherently diamagnetic [6].Recent

reports show that the possibility of the magnetism in graphene can be introduced by defects, adatoms or edge states [7-13]. The magnetic ordering due to defects has been attributed to the mixture of sp^2 - sp^3 bonding [7-11]. Spin half states at the edges of zigzag graphene [12] or a negative curvature in graphitic plane consisting of a few rings can introduce magnetic ordering [13]. Adatoms in graphene, like fluorine carry unpaired moments resulting in paramagnetic behavior [7].

Graphene confined to quantum dimension form stable, luminescent colloidal suspension known as graphene quantum dots (GQDs). Many theoretical predictions and calculations have been made towards the magnetism in graphene quantum dots [6,12,14-15]. Bhowmick *et. al.* showed the robustness of edge state magnetism in graphene nanostructures [12]. In a recent work by Sun *et. al.*, a weak paramagnetism is reported in graphene oxide quantum dots [16]. It has been observed that the edge states as well as the hydroxyl groups contribute to the paramagnetism. This paramagnetism in graphene due to edges and functional groups could be further enhanced by fluorine substitution in the lattice as reported by R.R Nair *et.al* [7]. In a recent work from our group, fluorinated graphene oxide (FGO) a novel material derived from fluorinated graphite polymer (FG) was reported to be paramagnetic [17]. This received a lot of attention as it was the first reported “magnetic nanoparticle free carbon based contrast agent for Magnetic Resonance Imaging (MRI)” [18].

MRI is a powerful and commonly used imaging tool, due to its high spatial resolution and non-ionizing radiations [19]. Gadolinium (Gd^{3+}) based contrast agents lead the market of MRI contrast agents in spite of their high inherent toxicity [20,21]. These agents are rapidly removed by renal clearance. As a result, the imaging window available with these agents is very short. This makes repeated administration ineludible for multiple imaging sessions [22]. The poor stability of

Gadolinium chelates release Gadolinium ions which accumulate in the brain causing long term functional and toxicological effects. Reports suggests that Gadolinium based agents can induce Nephrogenic Systemic Fibrosis(NSF) in patients with renal or vascular disorders [23]. Several studies in vitro, on human skin fibroblasts and proximal tubular epithelial cells also demonstrate the high toxicity of Gadolinium based agents [24,25]. Also these agents modify the contrast in an image. Therefore, for unambiguous detection of the localization of contrast agent in vivo, it is essential to have an image before and after injection of the agent. This is extremely difficult and laborious [26].

Other contrast agents based on Iron and Manganese has also garnered interest recently [22]. In Manganese based agents it is extremely difficult to achieve chelation stability. Some Manganese based contrast agents have shown detectable toxicity within two weeks of administration. Owing to these limitations only one manganese based agent, commercially available as Teslascan is approved for clinical use [23]. Iron oxide based agents confer negative contrast making it indispensable to have pre and post injection images for direct evaluation of the images [26].

These limitations call for an urgent and unmet need for a metal free contrast agent with less toxicity. FGO being metal free, is supposed to be less toxic for biological systems. However, the large size of these sheets limits its translation to in-vivo use as these will cut or block the capillaries proving to be fatal. Thus it is vital to study the magnetic behavior of fluoro graphene quantum dots having nanometric dimensions. Most importantly, these quantum dots could be developed as a dual modal agent for MRI and ^{19}F MRI. This would avoid the multiple imaging required while using Gd^{3+} based agents without compromising on efficiency.

There have been reports for dual modal agents for ultrasound and ^{19}F MRI [27], but this is presumably the first report of a metal free nanomaterial that could be used as a dual modal agent

for proton and ^{19}F MRI. The efforts in the development of a dual modal agent for ^{19}F and proton MRI has been focused on introducing Gd^{3+} ion into fluorinated materials. However, this relaxes the Fluorine faster making it unable to be detected by ^{19}F MRI [28]. To surmount these limitations, there is an urge to study the magnetic property of Fluoro graphene quantum dots. It could open up the doors for a metal free, cyto-compatible, dual modal agent with higher spatial resolution.

In this work we explore the magnetic behavior of fluoro graphene quantum dots prepared from FGO. The experimental observations are well supported by theoretical calculations. The structural and magnetic characterization of these quantum dots were carried out. The low temperature magnetic measurements do not show any signature of magnetic ordering. The temperature dependent susceptibility of FGO derived quantum dots follow Curie's law, which confirms their paramagnetic nature. DFT studies show that this paramagnetism arises due to fluorine adatoms and presence of functional groups. Each fluorine adatom and functional groups carry $1\mu_{\text{B}}$ (Bohr Magneton). This magnetic moment induces from the quasilocalized state, which is arising from the absorbed functional groups. The MRI relaxivity was measured to assess the relaxation rate and feasibility of these quantum dots as a contrast agent. The biocompatibility studies on human derived cell lines were carried out by MTS assay.

Results and Discussion

Fluoro graphene quantum dots (FGQd) were prepared from FGO by simple liquid exfoliation [29]. FGO was prepared by the oxidation of FG by an improved method [30]. The synthesis of FGO yields two products, a hydrophilic less fluorinated graphene oxide which settles to the bottom and a hydrophobic highly fluorinated graphene oxide which separates to the top. The highly fluorinated part, which separates to the top was then washed and used to prepare the quantum dots. The quantum dots were synthesized by a top down approach [29]. FGO was bath sonicated in N-N

Dimethyl formamide (DMF) for 6 hours. The hydrodynamic forces help to break down the larger flakes into smaller ones [31]. The top three quarter of the solution, which would contain the smaller flakes was decanted. This was stirred on a hot plate at 110°C for 6 hours. A disperse solution of the quantum dots and flakes in DMF was obtained. The quantum dots were separated by centrifugation at 10,000rpm for 30minutes. A schematic illustration of this synthesis is shown in figure 1.

The structural characterization of the quantum dots was carried out using Transmission Electron Microscope (TEM). The size varied from 8-12 nm while most of the dots were of the size~10nm. The size varied from 8-12 nm while most of the dots were of the size~10nm. The size measured by Dynamic Light Scattering (DLS) is shown in Figure S1 of the *Supporting Information*. DLS however indicates the size of the aggregates rather than individual particles. In the HRTEM shown in figure 2, a good superlattice structure with discoidal in shape can be seen. From the HRTEM, it can be inferred that FGQDs are single layered as a consequence of the rigorous exfoliation during the oxidation process combined with the exfoliation to quantum dots. The single layered structure obviates the possibility of spin pairing of fluorine in the adjacent layers.

Raman spectroscopy was used to get more structural information about the quantum dots. It shows many characteristics corresponding to high defect density graphene. The defects or the edges in graphene are capable of giving the D peak [32-35]. In the Raman spectra given in figure 2b the high D peak with an I_D/I_G ratio of 1.093 is arising from the increasing defects in the lattice due to the Fluorine doping and due to the higher number of edges in the quantum dots [36-38]. On comparison of the I_D/I_G ratio to that of graphene quantum dots [39,40] fluoro-graphene quantum dots have higher number of defects than graphene quantum dots. These are contributed by the sp^3 C atoms bonded to the fluorine. The G band seems to be upshifted from 1580cm^{-1} to 1600cm^{-1} . At

higher defect densities, the G and the D' band becomes too wide to form a single upshifted G band which is seen in this case [36]. The appearance of 2D peak which is a doublet peak of the D band and the D+D' band too is due to the high defects in the Quantum dots.

However, Raman spectroscopy does not give any information about the functional groups. Since the quantum dots are derived from the functionalized derivative FGO, there could be functional groups present. The presence of functional groups was verified from the IR spectrum (Figure 2c). The spectrum shows a characteristic C=O peak and a broad -OH peak showing the presence of acid functional groups. The entire spectrum shown in the inset indicates the presence of C-F bond. The optical property of the quantum dots studied using UV-Vis spectroscopy (Figure S2, *supporting Information*) shows the peak corresponding to π - π^* transition of C-C bond at 225nm. FG2 shows an additional peak corresponding to the n - π^* transition of carbonyl groups which confirms the presence of functional groups.

A qualitative analysis was carried out by X-ray Photoelectron Spectroscopy (XPS). The deconvoluted XPS spectrum is shown in Figure 3a. C1s spectrum peak at 284.1eV corresponds to the sp^2 carbon in graphene; 285.83eV agrees closely with that of ionic C-F bond. The F1s shows peak at 683.8eV which confirms the ionic nature of the bond with Carbon [37,38,41,42]. XPS analysis do not show the presence of any magnetic impurities like Iron. The XPS data shows that the relative fluorine content is 4.87%. It is reported that, at such low concentrations of fluorine, the carbon fluorine bond tends to be ionic, which is seen in this case [43].

A detailed magnetic characterization is performed to explore the magnetism in these fluorine doped quantum dots (figure 3c). The calculated susceptibility ($\chi=M/H$) with temperature (T) follows Curie's law ($\chi \propto 1/T$) as shown in figure 3d, which indicates fluorinated graphene quantum dots are purely paramagnetic nature. At low temperature, the inverse of susceptibility varies

linearly with temperature (Figure 3d inset), which also support that the samples have purely paramagnetic nature with no magnetic ordering. The M-H curve was measured at room temperature and the diamagnetic contribution of the background has been subtracted from measured magnetization (Figure 3c). FG shows a linear dependence of magnetization on the applied magnetic field. To further understand the magnetic properties of functionalized graphene quantum dots, we have modeled the real system by considering a 3x3x1 supercell of graphene in the first-principle density functional theory (DFT) calculations [44,45]. The details of the calculation are described in the theoretical method section. According to Lieb's second theorem, if the total number of electrons of a bipartite system is odd then the total magnetic moment of the system in the ground states has to be $M_S = \frac{\mu_B(N_\alpha - N_\beta)}{2}$ (where, $(N_\alpha - N_\beta)$ is the difference between the number of atoms in each sublattice), which is independent of dimensionality and periodic lattice structure. This theorem indicates that the ground state magnetic properties of graphene quantum dots will not be much difference from the periodic graphene lattice. The calculated total energy difference between magnetic and non-magnetic systems is shown in figure 4a. The magnetic solution of each functionalized graphene is lower in energy compared to the nonmagnetic state, which indicates that this functional group will introduce magnetism in the graphene. Each functional group contributes nearly $1\mu_B$ (Figure 4b), which is in well agreement with theoretical prediction [44]. To further understand the magnetism of functionalized graphene, spin charge density of functionalized graphene was calculated (Figure 4c-4e). The spin up charge density is localized around the functionalization group, which indicates that this magnetic center will behave like a paramagnet. This kind of charge localization is also observed in defected H and F functionalized graphene, which act as a paramagnetic center [7,46-49]. Moreover, the calculated bond length of C-F and C-O in F and OH functionalized graphene are 1.55 Å and 2.80 Å,

respectively. This indicates that F and OH functional group are covalently bonded with graphene carbon atom resulting in an sp^2 - sp^3 bonded atomic network, which is seen in the XPS analysis. The distance between C, O and H of COOH functional group and graphene carbon atom are 3.69 Å, 3.30-3.58 Å and 2.59 Å, respectively, which indicates that the COOH functional group is chemically adsorbed into graphene. Furthermore, the magnetic solution of OH and F functionalized graphene shows lower in energy when OH and F functional groups are adsorbed on the same sublattice of graphene. However, if OH and F functional groups are present in different sublattices of graphene, then non-magnetic solution is favored as shown in figure 4b. This finding is in good agreement with Leib's theorem [46]. The presence of both functional group slightly decrease the magnetic moment of the system as shown figure 4b. This suggest that the magnetic system of graphene is strongly depends on the concentration, presence of functional groups and absorption sites. Moreover, when OH and F functional groups are attached to neighboring carbon atoms of graphene, an unstable FOH molecule is formed, which results in a non-magnetic state. A stable CO₂ and HF molecule formation is found when COOH and F functional group is present in the graphene. This suggests that COOH functional groups have less chance to be on the surfaces of the graphene monolayer.

The magnetic behavior of a paramagnetic system is described by the Brillouin function as

$$M = NgJ\mu_B \left[\frac{(2J+1)}{2J} \coth\left(\frac{(2J+1)x}{2J}\right) - \frac{1}{2J} \coth\left(\frac{x}{2J}\right) \right] \quad \text{--- (1)}$$

Where $z = gJ\mu_B H/k$ and $x = (gJ\mu_B H/k_B T)$.

$M, n, g, J, \mu_B, H, T, k_B$ is the magnetization of the sample, number of spin(free), g -factor, the angular momentum, Bohr magneton, applied magnetic field, absolute temperature and Boltzmann constant, respectively. Since the DFT calculation also shows that each functional group contributes $1\mu_B$, the experimental M Vs H data is fitted with Eq. (1) by considering $J=S=0.5$ (for

free electron). The equation (1) is best fitted with n value $3.2 \times 10^{20} \text{ gm}^{-1}$, which is higher than the XPS measure the fluorine concentration $1.38 \times 10^{20} \text{ gm}^{-1}$. This discrepancy could be possible due to presence of other fictional groups and defects.

These magnetic centers in graphene, introduced by functional groups is observed in graphene oxide quantum dots [16]. However, the measured magnetization of the Graphene Oxide quantum dots starts saturating at 3T, while that of FGQds do not saturate upto the applied field of 5T. The paramagnetism along with this high value of saturation magnetism, could make these ideal agents for T2 contrast in MRI. In MRI imaging T2 measures the time required for the decay of the transverse magnetization induced by the RF pulse. The T2 relaxation was measured in a Bruker 4.7T, with 30 echo times from 10ms to 300ms. Pure water has a T2 relaxation of ~650ms. On the introduction of the quantum dots it reduced to 378ms. The plot showing the transverse magnetization decay is shown in *supporting information* Figure S3. The image acquired during MRI is shown in figure 5a. ^{19}F MRI is based on the relaxation of ^{19}F nuclei. Mobile fluorine is present in the body at very low concentrations which are below the detection limit. High concentration of fluorine is present in the bones which are immobilized and hence cannot be detected. Thus ^{19}F MRI has the advantage of very low background signal and the combination of proton and ^{19}F MRI eliminates the need for pre and post scans required for Gadolinium based agents [24]. ^{19}F MRI scans were conducted on the proton MRI system by modulating the resonance frequency. Signals could be picked up and an image could be generated at concentrations as low as $10 \mu\text{g/mL}$ (Figure 5b).

Calculations show that the relaxation rate r_2 for FGQds is 39 s/g/mL . The proposed mechanism of interaction of the protons and the paramagnetic agents is illustrated graphically in Figure 5c. The paramagnetic agents interact with the applied magnetic field and act as centers of local magnetic

field. This causes inhomogeneity in the magnetic field which interacts with the protons in water shortening their relaxation time. This value of relaxation is comparable to a commercial available, Gadolinium based contrast agent, Ablavar, previously marketed under the name MS 325 [50]. It is used for the evaluation of aortoiliac occlusive disease in adults with known or suspected peripheral vascular disease. An insight into the currently reported carbon based or graphene based contrast agents for MRI reveals that all of them are metal based agents. For instance, FeMn-GO with Iron and Manganese embedded in Graphene oxide [22] and Iron oxide-GO composites [51] have relaxivities in the order of 100mM/s. The toxicological issues associated with these metallic agents covers their high r_2 values.

A preliminary assessment of cytocompatibility is an indispensable part of this study. The cytocompatibility (Figure. 5d) of the quantum dots were measured by MTS assay. For the study, HEK 293T cells were incubated with the quantum dots for 1,2 and 3 days. At the end of the incubation time MTS reagent was added to the cells, and incubated for one more hour and the optical density was read with a micro plate reader at 490nm. The cells incubated in the growth media under identical environment was used as the control. The optical density reflects the number of viable cells. The results do not indicate any dose dependent toxicity. Even at the highest concentration no significant cell death is seen. Even after 3 days there is no considerable decrease in the viability, which rules out the possibility of incubation time dependent toxicity. The observation is further confirmed by cellular imaging GFP labelled SUM 159 cells were seeded at 1000cells/well and incubated with the quantum dots under identical conditions. A Zeiss axiovision A1 microscope with Axiovision software was used to capture the images after 1, 2 and 3 days of incubation. Figure 5e and 5f shows dark field fluorescent images of the cells after 1day and 3 days

of incubation respectively. The inset shows an overlapped image of the bright field and fluorescent image. The cells look viable and no proliferation defects are seen.

Conclusion

Magnetic Resonance Imaging (MRI) is a powerful imaging tool in the clinical biomedical imaging for disease diagnosis. It is based on the relaxation of protons in water due to the local magnetic field experienced by them. The “gold standard” contrast agents used today are Gadolinium or Iron based, which require post and pre images for efficient detection. Moreover, these metal based agents pose a threat of high toxicity with repeated administrations. The Fluoro graphene quantum dots synthesized from FGO possess novel attributes suitable for its use in imaging. The inherent paramagnetism with the presence of Fluorine enables its use as a dual modal agent for proton and ^{19}F MRI. This avoids the pre and post scan sessions required with conventional agents. The presence of functional groups and small size will enhance the chances of internalization of these agents into the tissues giving higher retention times and imaging efficiency than the conventionally used metallic contrast agents.

Experimental Section

Synthesis: Fluorinated graphene oxide was prepared by dispersing the Fluorinated Graphite polymer (FG) in a 9:1 mixture of concentrated sulfuric acid and concentrated phosphoric acid. The mixture was stirred at 50°C for 2 hours followed by the addition of 6gm equivalent of Potassium permanganate. On addition of potassium permanganate, the temperature of the mixture rises to 90°C . The mixture was left to stir overnight. The mixture was poured over ice and 10-12ml of hydrogen peroxide was added to it. On the addition of hydrogen peroxide, a light brown part settles on the bottom while a dark brown part separates to the top. The separation was allowed to complete by keeping the mixture undisturbed overnight. This observed phase separation is due to the

difference in fluorine content. The top portion is highly fluorinated which makes it hydrophobic, while the bottom portion is less fluorinated and hydrophilic part.

Magnetic measurements: The sample was sealed in vacuum in NMR tubes and the hysteresis curve was measured using a SQUID magnetometer at room temperature. Liquid samples were used for the measurement at the as prepared concentration. The background with the solvent was measured independently to eliminate the possibility of ferromagnetic impurities and was found to be diamagnetic.

Cyto-compatibility: MTS assay was used to assess the cyto-compatibility of the quantum dot samples. These studies were conducted by serial dilution of the quantum dots. HEK 293T cells cultured in DMEM cell culture media substituted with FBS and penicillin and Streptomycin was used for the studies. 1000 cells were seeded per well in a 96 well plate and the quantum dots were added to these with serial dilution. This was incubated at 37°C. After incubating it for 1 day, 2 days and 3 days the absorbance values were measured after the addition of MTS reagent. The fluorescent images were obtained using GFP labelled SUM 159 cells incubated under identical conditions.

MRI Relaxation time: The relaxation time measurements were conducted in a 4.7T Biospec system. The measurement was conducted by immersing the solution containing the quantum dots, sealed in ultra-small centrifuge in water with Magnevist serving as a positive control.

Theoretical methodology: All the theoretical calculations were performed using first-principle density functional theory (DFT) as implemented in the VASP package [46]. The exchange and correlation effects of electrons is approximated by using generalized gradient approximation Perdew-Burke-Ernzerhof (PBE) functional [47,48]. The frozen core approximation using projector-augmented wave (PAW) pseudo-potentials is used to represent the electrons and ion interaction [47]. The electronic wave functions are represented by the plane wave basis set with

400 eV energy cutoff. To model F, OH, COOH functionalized graphene system, a 3x3x1 graphene supercells having a 20 Å vacuum along the *c* axis is used in order to avoid any interaction between periodically repeated images. The reciprocal space was sampled by 5x5x1 Monkhorst-Pack *k*-point grids [50]. The final structures are relaxed unless the forces on each atom becomes less than 0.01 eV/ Å. It has been observed that the DFT theory within semi-local GGA approximation PBE functional studies of H and F functionalized graphene gives rise to a nonmagnetic states [53,54]. This semi-local GGA functional reduces delocalized electronic states due to their spurious inherent self-interaction error (SIE), which results to a nonmagnetic state of the functionalized graphene. The hybrid functionals such as PBE0 and HSE has shown good predictability of magnetic properties of functionalized graphene [55]. Here, we have employed the PBE0 functional, where exchange and correlation is approximated as follows [55].

$$E_{xc}^{PBE0} = E_{xc}^{PBE} + \frac{1}{4}(E_x^{HF} - E_x^{PBE}) \dots \dots \dots (3)$$

This Hybrid functional used 25% of HF with 75% of PBE exchange, which is orbital dependent i.e. non-local in space and greatly reduces the spurious inherent self-interaction error. The PBE relaxed structures are considered for the PBE0 functional calculations.

Supporting Information

Supporting Information is available online from the Wiley Online Library or from the author.

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Figure. 1. Schematic illustration of the top down method for the synthesis of fluorinated graphene quantum dots from Fluorinated Graphene Oxide (FGO). FGO is an oxidized derivative of Fluorinated Graphite having OH and COOH functional groups on the surface in addition to Fluorine atoms. It is further exfoliated by sonication and stirring at high temperature to break them down into quantum dots.

Figure. 2. a) Bright field Transmission Electron Microscope(TEM) image showing clusters of quantum dots, size distribution with a narrow distribution from 8-12nm, and High resolution TEM image of FGQds the marked area showing the lattice with the SAED pattern as inset confirming the crystalline nature b) Raman spectra of FGQds showing the characteristic G peaks associated with graphene and the defect peaks D, 2D and D+D' arising from the introduction of defects due to fluorination and functionalization c) FTIR spectrum of FGQds the peaks corresponding to the functionalities are marked. The inset shows the entire spectrum showing the presence of C-F bond.

Figure. 3. Deconvoluted XPS spectrum a) C1s spectra of FGQds showing sp² C and C-F bonds b) F1s spectra of FGQds with a single peak corresponding to the C-F bonding c) SQUID hysteresis curve for FGQds, the linear dependence of the Magnetization on the applied field showing paramagnetic behavior is seen d) ZFC curve showing the experimental and fitted curves. The inset shows the curie's law dependence showing the linear dependence of inverse of susceptibility on temperature confirming the paramagnetism.

Figure. 4. a) Energy difference (ΔE) between total energy of magnetic and nonmagnetic solution of the functionalized graphene b) Total magnetic moment of the functionalized graphene. c) d) and e) Spin charge density plot of F, OH and, COOH functionalized graphene respectively. The

spin up and spin down charge density is represented by blue and yellow color respectively. The C, F, O and H atoms is depicted by cyan, red, magenta and blue color balls, respectively.

Figure. 5. a) The image of a slice acquired during the proton MRI showing the contrast where the paramagnetic FGQDs are present b) Image acquired during ^{19}F MRI showing the relaxation of ^{19}F nuclei giving a contrast c) schematic showing the mechanism of relaxation of protons in water by the quantum dots. When the RF pulse is applied the local magnetic field created by the quantum dots interact with the protons in water shortening of the T_2 relaxation time d) The results of MTS assay of the HEK 293T cells incubated with the quantum dots at different concentrations for 1, 2 and 3 days e) Images of GFP labeled SUM 159 cells incubated with the highest concentration of quantum dots after 1-day f) After 3days of incubation, inset shows an overlapped bright field and fluorescent image.

Figure.1

Figure.2

Figure.3

Figure.4

Figure.5

The table of contents entry:

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Metal Free Dual Modal Contrast Agents based on Fluorographene Quantum Dots

TOC: Schematic shows a representative of the systems of fluoro-graphene quantum dots studied here. Non-functionalized and functionalized fluoro-graphene systems are studied of which acid functionalized system shows greater magnetic moment. This is experimentally verified and the hysteresis and low temperature studies reveal paramagnetic behaviour. The inherent paramagnetism along with the fluorine content is utilized in its development as a dual modal agent for proton and ¹⁹F MRI.