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Carbon and Silicon Nanomaterials for Medical Nanotechnology Applications

by

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ABSTRACT

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This dissertation focuses on the development of sp²-carbon- and silicon-based nanomaterials for medical diagnostics and in vivo magnetic field-guided delivery applications. To realize these applications, especially for the development of new in vivo Magnetic Resonance Imaging (MRI) contrast agents (CAs), high solubility in aqueous media is required. Therefore, this work first details development of a new non-covalent method for the preparation of stable aqueous colloidal solution of surfactant-free sp²-carbon nanostructures, as well as a second rapid covalent functionalization procedure to produce highly-water-dispersible honey-comb carbon nanostructures (ca. 50 mg/mL). Next, highly-water-dispersible graphene nanoribbons and Gd³⁺ ions were together used to produce a high-performance MRI CA for T₁- and T₂-weighted imaging. In terms of its relaxivity (r₁₂) values, this new CA material outperforms currently-available clinical CAs by up to 16 times for r₁ and 21 times for r₂. Finally, sub-micrometer discoidal magnetic nanoconstructs have been produced and studied for applications for in vivo magnetic-field-guided delivery into cancerous tumors. The nanoconstructs were produced by confining ultra-small superparamagnetic iron oxide nanoparticles (USPIOs) within mesoporous silicon which produced T₂-weighted MRI CA
performance 2.5 times greater than for the free USPIOs themselves. Moreover, these nanoconstructs, under the influence of an external magnetic field, collectively cooperated via a new mechanism to amplify accumulation in melanoma tumors of mice. Overall, the results of this dissertation could aid in the rapid translation of these nanotechnologies into the clinic, while, hopefully, also serving as an inspiration for continued research into the field of Medical Nanotechnology.
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estimated at 0, 4 and 24 h post injection. i–k) MR images of a melanoma tumor growing in the right flank of a mouse before, 4 h, and 24 h post injection of magnetic nanoconstructs, in the presence of a static magnet applied over the tumor. l) Intensity ratios for two ROIs estimated at 0, 4, and 24 h post injection. Note that the intensity ratios have been calculated by averaging the MRI signal over multiple z-planes. A 3T Philips MRI clinical scanner was used. B16-F10 cells were grown in the flank of a mouse for 10–15 days prior injection of $5.0 \times 10^8$ nanoconstructs via tail vein. This dose corresponds to $\approx 8 \ \mu g$ of SPIOs per mouse (i.e., $\approx 0.5 \ mg$ of Fe kg$^{-1}$).

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Preface

The Chapters of this Dissertation are self-contained and can be read in any order.

First, stable aqueous colloidal solutions of graphene nanoribbons and carbon nanotubes have been prepared by a simple and nondestructive methodology. That includes brief sonication of carbon nanomaterials in hypophosphorous acid, washing steps with water and dispersion of the solids to form aqueous colloidal solutions. The procedure does not employ surfactants and does not modify the sp²-hybridization of the graphitic nanostructures.

Second, highly water-dispersed and stable multi-layer graphene nanoribbons (4.7 mg/ml) have been prepared for the first time by repetitious derivatization using a p-carboxyphenylidiazonium salt. Using a similar methodology, single-walled carbon nanotubes (4.8 mg/ml) and ultra-short carbon nanotubes (50 mg/ml) can also be made highly water-dispersible. The methodology used to functionalize these carbonaceous materials is fast, easily scalable, and uses an aqueous procedure.

Third, a high-performance MRI contrast agent has been synthesized by conjugating aquated Gd³⁺ ions to the surface of surfactant-free highly water-dispersed graphene nanoribbons. The resulted material can be used for T₁- and T₂-weighted imaging and it outperforms currently-available clinical Gd³⁺ ion-containing contrast agents by up to 16 times for r₁ and 21 times for r₂.
Fourth and finally, hierarchically structured magnetic nanoconstructs have been prepared by confining 5 nm ultra-small superparamagnetic iron oxide nanoparticles (USPIO)s within sub-micron sized discoidal mesoporous silicon particles (SiMP)s. The resulting nanoconstructs have showed transversal relaxivities up to 2.5 times higher that of free USPIOs, and under the influence of an external magnetic field, a new cooperative accumulation mechanism within the melanoma tumor of mice has been demonstrated and visualized via MRI for the first time.
<table>
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<tr>
<td>°C</td>
<td>Degree Celsius</td>
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<tr>
<td>CA</td>
<td>Contrast Agent</td>
</tr>
<tr>
<td>CNS</td>
<td>Carbon Nanostructure</td>
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<tr>
<td>CNT(s)</td>
<td>Carbon Nanotube(s)</td>
</tr>
<tr>
<td>CPMG</td>
<td>Carr-Purcell-Meiboom-Gill</td>
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<td>DI</td>
<td>Deionized</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<td>EDS</td>
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<td>LTO</td>
<td>Low Temperature Oxide</td>
</tr>
<tr>
<td>MD</td>
<td>Molecular Dynamics</td>
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</table>
MFM  Magnetic Force Microscopy
MRI  Magnetic Resonance Imaging
MSD  Mean Square Displacement
MWCNT(s)  Multi-walled CNT(s)
NMR  Nuclear Magnetic Resonance
PB  Prussian Blue
PBS  Phosphate Buffered Saline
PCP  p-carboxyphenylidiazonium
PEG  Polyethylene glycol
PME  Particle Mesh Ewald
PMMA  Poly(methyl methacrylate)
POM  Polarizing Optical Microscope
r₁ or r₂  Relaxivity
RIE  Reactive Ion Etch
ROI  Region of Interest
RT  Room Temperature
SEM  Scanning Electron Microscopy
SiMP(s)  Sub-micron Sized Mesoporous Silicon Particle(s)
SPIO(s)  Superparamagnetic Iron Oxide Nanoparticle(s)
SWCNT(s)  Single-walled Carbon Nanotube(s)
T₁,₂  Relaxation
TE  Echo Time
TEM  Transmission Electron Microscopy
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>TGA</td>
<td>Thermal Gravimetric Analysis</td>
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<tr>
<td>TI</td>
<td>Inversion Time</td>
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<td>TSE</td>
<td>Turbo Spin Echo</td>
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<tr>
<td>USPIO(s)</td>
<td>Ultra-small Superparamagnetic Iron Oxide Nanoparticle(s)</td>
</tr>
<tr>
<td>US-tube(s)</td>
<td>Ultra-short Single-walled Carbon Nanotubes</td>
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<td>X-ray photoelectron spectroscopy</td>
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<td>XRD</td>
<td>X-ray Diffraction</td>
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Chapter 1

Stable aqueous colloidal solutions of intact surfactant-free graphene nanoribbons and related graphitic nanostructures\(^1\)

1.1. Introduction

Dispersion of carbon nanotubes (CNTs) and graphene nanoribbons (GNRs) in liquid media is a crucial step for numerous applications. The properties of dispersions are highly dependent upon the nature of the material and the dispersing media interface. All graphitic carbon nanostructures are highly hydrophobic. Due to this hydrophobicity, they do not form stable uniform dispersions in aqueous media.

\(^1\) This chapter has been published in the following research article: Dimiev, A.; Gizzatov, A.; Wilson, L. J.; Tour, J. M. Stable aqueous colloidal solutions of intact surfactant-free graphene nanoribbons and related graphitic nanostructures. *Chem. Commun.* **2013**, *49*, 2613-2615 - Reproduced by permission of The Royal Society of Chemistry.
At the same time there is growing interest in applications that require water-based dispersion for biomedical engineering and to promote ecologically friendly processes.\textsuperscript{1–3}

There are two major methods developed to introduce CNTs into aqueous media: chemical modification to introduce water-solubilizing functional groups and the use of surfactants to wrap the CNTs thereby making the surfaces hydrophilic. Chemical modification, which includes oxidation\textsuperscript{4} or covalent functionalization with diazonium salts\textsuperscript{5,6} introduces numerous sp\textsuperscript{3}-hybridized sites into the CNT structure, leading to a major alteration of its electronic properties. The use of surfactants is not destructive to the CNTs, however, in this case an excessively thick layer of surfactant accumulates on the surface of the CNTs.\textsuperscript{7–10} The thickness of the surfactant layer is comparable to, and in many cases is thicker than, the diameter of the CNT. The resulting surfactant-wrapped CNT can be considered a surfactant micelle with a CNT core. Thus, the use of surfactants is not preferred when the purity of the CNTs and the nature of the CNT/water interface is important.

Here we demonstrate a new method to prepare stable colloidal solutions of GNRs and multi-walled CNTs (MWCNTs) in aqueous media that does not destroy the sp\textsuperscript{2}-hybridization and that does not involve formation of a thick layer of surfactant on the surface. The method includes bath sonication of the carbon structures in 50 wt\% hypophosphorous acid (H\textsubscript{3}PO\textsubscript{2}) for 2–3 min and subsequent washing of the solids with water. The resulting H\textsubscript{3}PO\textsubscript{2}-modified carbon nanostructures (HP-CNSs) can be easily redispersed in deionized water by mild bath sonication, forming a
stable colloidal solution. The observed phenomena can be explained by adsorption of a monomolecular layer of H$_3$PO$_2$ on the CNS surface with subsequent dissociation of adsorbed H$_3$PO$_2$ molecules upon washing and redispersion of HP-CNSs in pure water. Dissociation of H$_3$PO$_2$ molecules generates negative charge on the surface, which facilitates the stability of the colloidal solution.

1.2. Materials and Methods

1.2.1. Materials

The MWCNTs were purchased from Mitsui & Co. and were used without further treatment. The splitting of MWCNTs was performed as we described earlier.$^{11,12}$ The 50 wt% hypophosphorous acid was purchased from Sigma-Aldrich. The water was HPLC grade from Sigma-Aldrich.

1.2.2. Functionalization and preparation of colloid solutions

30 mg of GNRs (or MWCNTs) were added into 30 mL of 50 wt% H$_3$PO$_2$ and sonicated for 2-3 min using a bath sonicator. The resulting dispersion was filtered through the porous membrane (4 μm pore size) with suction. For washing, the filter cake was first rinsed with ~100 mL water on the filter. Next the wet filter cake was collected from the filter and redispersed in 100 mL of water with sonication. The dispersion was filtered again to separate modified GNRs (HP-GNRs) from the washing water. These redispersion-filtration cycle was repeated one more time. To prepare colloidal solutions, the purified HP-GNRs were collected from the filter
while still wet and redispersed in water with mild sonication in cup sonicator for 20 – 30 s.

1.2.3. Characterization

UV-Vis absorbance spectra were acquired using a Shimadzu UV-3101PC UV-Vis-NIR scanning spectrophotometer. The ζ-potentials were measured using a Malvern Zen 3600 Zetasizer based on the Smoluchowski equation.13

1.3. Results and Discussion

The GNRs used here were prepared by chemically splitting MWCNTs using K or Na/K alloy as described elsewhere.11,12 The length of the parent Mitsui MWCNTs was 8–20 μm, and the diameter was 40–90 nm. The multilayer GNR stacks derived from these MWCNTs have a width of 125–280 nm and a thickness of 7–15 nm (Figure 1.1). According to the IUPAC classification, suspensions of solids with a particle size <1 μm in at least one dimension are considered colloidal solutions.13 The GNRs and MWCNTs are both far below the 1 μm limit in two of their three dimensions. From this perspective, the prepared GNRs and MWCNTs dispersions can be classified as colloidal solutions even though their length exceeds 1 μm.
Figure 1.1. SEM images of (a) Mitsui-MWCNTs (b) Mitsui-MWCNT-derived GNRs.

Figure 1.2 a and b shows that HP-GNRs form a stable dispersion in water, while unmodified GNRs (Figure 1.2 c and d) do not. As evident from Figure 1.2 b, the vast majority of HP-GNRs in solution are dispersed, while unmodified GNRs exist only as aggregates (Figure 1.2 d).
Figure 1.2. (a) A photograph and (b) an optical microphotograph of an aqueous colloid solution of HP-GNRs in deionized water. (c) A photograph and (d) an optical microphotograph of an unmodified (non-H$_3$PO$_2$-treated) GNR dispersion. The content of GNRs in both samples was 0.2 mg/mL. Photographs (a) and (c) were taken 1 h after dispersing the GNRs in water.

To quantify the solution stabilities, the coagulation rates were measured using UV-Vis spectroscopy (Figure 1.3). The HP-GNR colloidal solutions absorb in the entire range of 200–800 nm with a well-pronounced maximum at 272 nm, corresponding to the absorbance of graphene$^{14}$ (Figure 1.3 a). The measured absorbance at any wavelength taken in the 270–800 nm range was directly proportional to the solution concentration (Figure 1.4). This behavior, typical for true solutions, additionally confirms the uniformity of the HP-GNR colloid solutions. The absorbance of the dispersion of unmodified GNRs is due to the light scattering
from the aggregates suspended in water (Figure 1.2 c). These aggregates quickly precipitated within a few hours after dispersion; the absorbance decreased proportionally (Figure 1.3 b). The absorbance of the HP-GNR solution is significantly higher and the coagulation rate is relatively low. After 7.5 h and 48 h of standing, the solution still contained 96% and 78%, respectively, of the originally dispersed HP-GNRs (Figure 1.3 b). Considering the size of the HP-GNRs, the solution stability is remarkably high. The curve labeled as “NaHP-GNR” (Figure 1.3 b) refers to a solution made from the GNRs modified with sodium hypophosphite (NaH$_2$PO$_2$), a salt of H$_3$PO$_2$. As evident from the curve in Figure 1.3 b, NaHP-GNRs form a fairly stable colloid solution upon sonication but they are nearly completely coagulated within 1 d.

![Figure 1.3](image)

**Figure 1.3.** The UV-Vis absorbance spectra for GNR aqueous dispersions. (a) Absorbance of the HP-GNR colloid solutions at concentrations from 0.025 to 0.2 mg/mL. (b) Coagulation rates for 0.2 mg/mL aqueous dispersions of HP-GNRs, GNRs, and NaHP-GNRs. The absorbance was measured at 530 nm. “Zero time” corresponds to measurements performed within 5 min after dispersion by sonication.
The colloid stability is most likely due to the electric charge formed on the graphitic surface during the H$_3$PO$_2$-modification. The amount of electrical charge on the colloid particles can be quantified by measuring the Z-potential ($\zeta$). The $\zeta$ values were derived from particle electrophoretic mobilities based on the Smoluchowski equation.$^{13}$ The 0.1 mg/mL HP-GNR solution used for the measurements had pH 7.69 and $\zeta = -62.5$ mV (Figure 1.5 a). Colloidal solutions with $|\zeta|$ values between 40 mV and 60 mV are considered stable, and colloids with $|\zeta|$ values higher than 60 mV are considered very stable.$^{13}$ Thus, based on the $\zeta$ values, the HP-GNR colloidal solutions are classified as very stable. Addition of an acid to the original solution gradually decreases the $|\zeta|$, and the zero charge point is reached at pH $\approx$ 2.8. Further acidification does not result in building positive charge on the GNRs, and $\zeta$ values stay $\approx$ 0 mV. Rapid coagulation occurs at pH $\approx$ 4.8. Addition of a strong base to the

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**Figure 1.4.** Absorbance of as-prepared HP-GNRs colloid solutions with different GNRs content. The absorbance values are taken at 272 nm.
original HP-GNR solution gradually increases $|\zeta|$ values, which reach −83 mV at pH = 11.69. This is an extremely high $|\zeta|$ value for colloidal particles. Thus, the solution stability can be additionally increased by addition of strong base. It was found that at pH ≈ 10 the coagulation rate decreases ≈ 2 times compared to the original solution with pH ≈ 8. At high ionic strength (0.1 M NaCl), $|\zeta|$ decreases (Figure 1.5 b). This likely occurs due to more effective screening of the surface charge by the counterions.

![Figure 1.5. $\zeta$ of HP-GNRs at 0.1 mg/mL in water. (a) $\zeta$ as a function of pH of the HP-GNR colloidal solution. The pH of the solution was tuned by addition of 0.1 M HCl or 0.1 M NaOH to the original solution. The black arrow indicates the pH of the original HP-GNR colloidal solution. (b) $\zeta$ values at pH 7.7 – 11.6 at different solution ionic strength values.](image)

Characterization of solid HP-GNRs did not show any difference from the unmodified GNRs. The Raman spectra and the powder X-ray diffraction (XRD) data for the two are identical (Figure 1.6 and Figure 1.7), suggesting no intercalation and no covalent functionalization takes place. The X-ray photoelectron spectroscopy (XPS) data shows the presence of only 0.4–0.6% phosphorus in the HP-GNRs. The
scanning electron microscopy (SEM), transmission electron microscopy (TEM), and high resolution transmission electron microscopy (HRTEM) of HP-GNRs (Figure 1.8) did not reveal any detectable structures adsorbed on the graphitic surface.

![Raman spectra of GNRs and HP-GNRs.](image)

**Figure 1.6.** Raman spectra of GNRs and HP-GNRs.
Figure 1.7. X-Ray diffraction data for GNRs and HP-GNRs.

Figure 1.8. (a) SEM, (b) TEM, and (c) HRTEM images of HP-GNRs made from Mitsui MWCNTs.
H₃PO₂ is a moderately strong acid with pKa = 1.23. According to this pKa value, the 50 wt% aqueous H₃PO₂ used for modification was 7.7% ionized. It is likely that the neutral acid molecules are adsorbed on the graphitic surface with formation of a monomolecular layer; the process is represented in Equation 1.1.

\[ \text{GNR} + n\text{H}_3\text{PO}_2 \rightarrow \text{GNR}/(\text{H}_3\text{PO}_2)_n \]

**Equation 1.1. Formation of a monomolecular layer.**

The surface-adsorbed H₃PO₂ molecules are held by strong specific adsorption forces and can be considered a part of the GNR surface. During the washing procedures, when the H₃PO₂ in the bulk solution is washed away, a part of the surface-adsorbed H₃PO₂ ionize by reacting with water according to Equation 1.2.

\[ \text{GNR}/(\text{H}_3\text{PO}_2)_n + m\text{H}_2\text{O} \Leftrightarrow \text{GNR}/(\text{H}_3\text{PO}_2)_{n-m}(\text{H}_2\text{PO}_2^-)_m + m\text{H}_3\text{O}^+ \]

**Equation 1.2. Partial ionization of the surface-adsorbed H₃PO₂.**

Ionization generates negative electrical charge on the GNR surface which facilitates formation of an electrical double layer. In solution the GNR/(H₂PO₂⁻)ₙ structure is likely surrounded by a diffuse layer of hydronium cations which neutralize the surface negative charge. The removal of hydronium cations with repeated washing causes the equilibrium in Equation 1.2 to shift to the right, resulting in gradual increase of the surface negative charge. Part of H₂PO₂⁻ ions likely desorb from the surface due to very high surface charge density. When solid
HP-GNRs are separated from the washing water by filtration, the entire diffuse layer is stripped away from the GNR/(H$_2$PO$_2^-$)$_n$ surface. Only the internal Helmholtz layer of solvent, containing cations, likely remains at the GNR/(H$_2$PO$_2^-$)$_n$ surface. When the stripped HP-GNRs filter cake is redispersed in a fresh portion of water, the negatively charged GNR/(H$_2$PO$_2^-$)$_n$ structure acts as a Brönsted–Lowry base and abstracts protons from surrounding water molecules according to Equation 1.3.

$$\text{GNR}/(\text{H}_2\text{PO}_2^-)_n + m\text{H}_2\text{O} \rightleftharpoons \text{GNR}/(\text{H}_2\text{PO}_2^-)_{n-m}\text{(H}_3\text{PO}_2)^m + m\text{OH}^-$$

**Equation 1.3. Formation of hydroxide anions.**

Formation of hydroxide anions in this reaction explains the basic character of the as-prepared colloidal solutions. The 0.1 mg/mL HP-GNR solutions have a pH in the range 7.5–8.0, and the 0.2 mg/mL solutions have pH > 8.0. These are very high values, considering that the wash water has pH 6.0–6.5 due to dissolved carbon dioxide. The H$_2$PO$_2^-$ ion by itself, being an extremely weak base with pKb = 12.77, cannot account for the basic character of the HP-GNR solutions. Unlike the single H$_2$PO$_2^-$ ion, the GNR/(H$_2$PO$_2^-$)$_n$ structure is a much stronger base due to its high negative charge density.

In a control experiment, the pristine MWCNTs were treated with H$_3$PO$_2$. The resulted H$_3$PO$_2$-modified MWCNTS (HP-CNTs) also form colloidal solutions in water, but with a lower concentration of dispersed HP-CNTs compared to the use of HP-GNRs. The highest solution concentration of HP-GNRs was 0.2 mg/mL, while for HP-CNTs value is 0.16 mg/mL. The GNRs have a surface area twice that of the parent
MWCNTs, assuming the former are completely split. Subsequently, GNRs can adsorb twice as many H₃PO₂ molecules, and can carry more electrical charge. Interestingly, HP-CNTs have measured |ζ| values (≈ 60 mV) only slightly lower those of HP-GNRs. This is likely because the ζ values are calculated based on measured mobilities and GNRs have a lower mobility in water compared to CNTs due to higher surface area. Thus, the comparison of ζ for particles with different shapes is not entirely informative. The same limitations can be taken for the coagulation rates. Nevertheless, the experiment with pristine MWCNTs demonstrated that the method can be applied to other graphitic carbon nanostructures, as well.

The Mitsui MWCNTs used in experiments described above are relatively large in size. Due to gravitation force and strong Van der Waals interactions, the solution coagulation rates were relatively high (Figure 1.3 b). One might expect that for smaller MWCNTs and their resulting GNRs, the solutions would be more stable. This is indeed the case. In a control experiment, Baytube MWCNTs were modified with H₃PO₂. Baytube MWCNTs have a length of 1–4 μm and diameter of 10–40 nm (Figure 1.9). Their average volume, weight and associated force of gravity are roughly 40 times smaller than those of Mitsui MWCNTs. The colloidal solutions prepared from Baytube-based HP-GNRs and HP-CNTs showed remarkable stability. Thus, the solutions with concentrations of 0.1 mg/mL did not show any signs of coagulation after five months of standing. This experiment demonstrates that extremely stable aqueous colloidal solutions can be prepared using this method.
1.4. Conclusions

To summarize, a simple and nondestructive method for the preparation of stable aqueous colloidal solutions of GNRs and CNTs has been developed. The colloidal solutions are stabilized by a negative charge built up on the graphitic surface due to specific adsorption of $\text{H}_3\text{PO}_2$ molecules and their subsequent ionization. The experimental data for coagulation rates and the character of $\zeta$ as a function of pH suggests that the resulting dispersions behave as typical lyophobic colloidal solutions. Due to the absence of surfactants on the graphitic surface, the method could find utility in a number of applications ranging from transparent conductive films to biological systems where both the solubility in aqueous media and the surface purity are critical.
Chapter 2

Highly water soluble multi-layer graphene nanoribbons and related honey-comb carbon nanostructures²

2.1. Introduction

Research involving honey-comb carbon nanostructures such as graphene and carbon nanotubes is a promising area both in chemistry and material science.²,¹⁵,¹⁶ Due to their unique combination of thermal, mechanical, chemical and electronic properties these nanomaterials are proving important for future applications in electronics,¹⁷ composite materials¹⁸ and nanomedicine.¹⁹

² This chapter has been published in the following research article: Gizzatov, A.; Dimiev, A.; Mackeyev, Y.; Tour, J. M.; Wilson, L. J. Highly water soluble multi-layer graphene nanoribbons and related honey-comb carbon nanostructures. Chem. Commun. 2012, 48, 5602-5604 - Reproduced by permission of The Royal Society of Chemistry.
Carbonous nanomaterials such as those reported here are particularly attractive as candidates for carbon fiber spinning,\textsuperscript{15,20} for the development of conductive polymer composites\textsuperscript{18} and as fillers in polymer matrices.\textsuperscript{21} To realize such applications, carbonous nanomaterials must be highly and easily soluble (dispersible) in solution, and aqueous dispersions offer a greener approach for large scale production. In addition, aqueous dispersions of such materials are needed to further realize their potential in nanomedicine where high solubility can be required.\textsuperscript{22,23} Even though many other diazonium salt methods have been developed to solubilize carbonous nanomaterials,\textsuperscript{24–28} most of these methods are impractical for applications where short reaction times and extremely high solubility in aqueous solutions are required. Thus, a convenient, rapid and universal aqueous method to solubilize these carbonous nanomaterials in bulk quantities remains highly desirable. Here we describe such a method for the bulk chemical functionalization of honey-comb carbon nanostructures such as multi-layer graphene nanoribbons (GNRs),\textsuperscript{4} single-walled carbon nanotubes (SWCNTs)\textsuperscript{29} and ultra-short single-walled carbon nanotubes (US-tubes).\textsuperscript{30} The chemical method used to functionalize these carbon nanostructures is direct, fast and easily scalable, and it employs an aqueous procedure.
2.2. Materials and Methods

2.2.1. Preparation of the carbonous nanomaterials

GNRs were prepared by melting K over multiwalled carbon nanotubes (Mitsui & Co.) under vacuum (0.05 Torr) as it was reported somewhere else.\textsuperscript{11} SWCNTs were purchased from Carbon Solutions Inc. and used as received. US-tubes were chemically cut by previously established methodology.\textsuperscript{30} Briefly, 200 mg of single-walled carbon nanotubes (Carbon Solutions Inc.) were fluorinated using 2\% F\textsubscript{2} in He gas mixture with a flow rate adjusted to 15 cm\textsuperscript{3}/min along with H\textsubscript{2} gas at a flow rate of 10 cm\textsuperscript{3}/min at 125 °C for 2.5 h. The fluorinated product was then heated to 1000 °C for 2 h. under continuous flow of Ar. Produced US-tubes were sonicated in 200 mL of concentrated HCl for 15 min to remove metal impurities.

2.2.2. Functionalization of the carbonous nanomaterials

In a typical reaction, the carbonous nanomaterial (35 mg) was added to a flask equipped with a magnetic stirring bar, and 30 ml of DI water was added. The solution was then sonicated for two minutes and 500 mg of p-carboxyphenyldiazonium (PCP) tetrafluoroborate (prepared according to a literature procedure)\textsuperscript{31} was added. The reaction was initiated by addition of 50\% w/w hypophosphorous acid in water (15 ml), and the solution was left to stir at RT for 10 min. The resulting suspension of the product was then neutralized with NaOH (aq.), briefly sonicated, and collected by centrifugation for two min at 1200 G. The washing steps were then repeated twice using 10 ml of acetonitrile, followed by 10
ml water to remove unreacted $p$-carboxyphenyl diazonium tetrafluoroborate. This derivatization step needed to be repeated three more times to optimize functionalization and solubility, a fact which apparently has not been previously appreciated for diazonium salt derivatization of carbon nanostructures. After each step, part of the product was collected for characterization.

2.2.3. Solubility of the carbonous nanomaterials

Solubility of functionalized US-tubes determined as described in literature. The solubilities of functionalized GNRs and functionalized SWCNT were estimated as described in literature by dispersing excessive amounts (12 mg) of the nanomaterial in 2 ml water. The resulting dispersions were left to stand for 5 hours and 400 µl of each was carefully taken from the upper part of the solution with a micropipette and evaporated in high vacuum at -50 °C. The residues were then weighed, from which the concentrations for functionalized GNRs and functionalized SWCNTs were calculated as 4.7 mg/ml and 4.8 mg/ml, respectively.

2.3. Results and Discussion

The highly-efficient aqueous procedure for functionalizing carbon nanomaterials was achieved using multiple $p$-carboxyphenyl diazonium tetrafluoroborate reduction reactions of Figure 2.1, which covers the honeycomb carbon surface with many carboxylic acid groups. The carboxylic acid groups are separated from the surface of the carbonous nanomaterial by phenyl groups acting as rigid spacers to produce highly water soluble structures with excellent aqueous
stability at pH 6.5–11.5 (Figure 2.2). For example, a maximum concentration for such functionalized US-tubes (PCP-US-tubes) in water at pH 7.9 is as high as ca. 50 mg/mL (Figure 2.3). Similarly, estimated maximum concentrations in aqueous solutions for PCP-GNRs and PCP-SWCNTs are as high as 4.7 mg/ml and 4.8 mg/ml, respectively. The \( p \)-carboxyl group of Figure 2.1 might also serve as a reactive functional group for further derivatization to achieve enhanced biocompatibility or even solubility in solvents other than water.

Figure 2.1. Aqueous phase functionalization of GNRs, SWCNTs and US-tubes with \( p \)-carboxyphenyldiazonium tetrafluoroborate.
Figure 2.2. Aqueous colloidal solutions of PCP-GNRs at 2 mg/ml versus GNRs at 2 mg/ml a) 15 min after sonication, b) 3 h after sonication, c) 1 month after sonication, d) aqueous colloidal solutions of PCP-GNR, PCP-SWCNT, and PCP-US-tubes at 0.1 mg/ml, where the PCP-GNR solution appears darker compared to the PCP-SWCNT and PCP-US-tube solutions due to the larger size of the PCP-GNR particles, e) aqueous solution of the PCP-US-tubes at 14 mg/ml, f) Tyndall effect (with 640-660 nm laser beam and maximum output of >1 mW) demonstrating a fine suspension of the PCP-GNRs in water.
Figure 2.3. A Beer’s Law plot for an aqueous solution of PCP-US-tubes-4 at 928 nm with a light path length of 0.05 mm.

The diazonium salt reduction by hypophosphorous acid (or other reducing agents),
first documented by J. Mai, has been recently applied to functionalize multi-walled carbon nanotubes, but not to solubilize them. Here we show that the reaction of Figure 2.1, if performed repeatedly (4–5 times) upon these carbonous nanomaterials, produces highly water stable colloidal solutions in only 1–2 h of work-up time. With such short reaction times, it should be possible to functionalize
even time-sensitive materials, such as radionuclide@US-tube species for nuclear medicine applications.\textsuperscript{36}

The carbonous materials used in this work were obtained as described in materials and methods part. Derivatization of the carbonous nanomaterials was accomplished according to Figure 2.1. The resulting carbonous materials all showed high solubility and stability in water for more than one month (Figure 2.2).

The derivatized materials were characterized by Transition Electron Microscopy (TEM), Polarizing Optical Microscope (POM), Thermal Gravimetric Analysis (TGA), Raman spectroscopy, X-ray photoelectron spectroscopy (XPS), and zeta potentiometry.

TEM images (Figure 2.4) showed that the structure of the US-tubes was preserved after repeated functionalization. Aqueous solutions of the GNRs and the PCP-GNRs were imaged using optical microscope (Figure 2.5). From the figure, it is clear that the functionalization procedure greatly disperses the GNR aggregates. GNRs of such a large size (up to 10 μm in length) have not been previously reported to show a highly-stable dispersion in aqueous solution.
Figure 2.4. TEM images of a) the US-tubes and b) the PCP-US-tubes-4 (both scale bars = 10 nm).

Figure 2.5. POM images of an aqueous dispersion of a) the GNRs and b) the PCP-GNRs.

Since extensive heating of honey-comb carbonous nanomaterials can remove covalently attached organic moieties to restore the pristine structure,$^{37}$ TGA has been used to determine the degree of functionalization for the PCP-GNRs, PCP-SWCNTs, and PCP-US-tubes. A two-step decomposition was clearly observed for the
PCP-SWCNTs and PCP-US-tubes (Figure 2.6 and Figure 2.7). The first major weight loss for both materials took place in the range 220–300 °C, probably as a result of decarboxylation. The second step between 300–460 °C likely corresponds to the loss of the benzene moiety. The observed combined percent weigh loss for the PCP-GNRs was less than for the PCP-SWCNTs or PCP-US-tubes (Figure 2.8, Figure 2.6, Figure 2.7), which is expected due to the multi-layer and less strained structure of the graphene ribbons compared to the tubular and single-layer structure of the SWCNTs and US-tubes. TGA profiles of the repeatedly-functionalized GNR and US-tube materials (Figure 2.8 and Figure 2.7) demonstrated that the degree of functionalization increases as the derivatization reaction is repeated step-wise. Hence, repetitive functionalization increased the degree of carboxylic acid groups on the surface of the carbonous nanostructure, and subsequent conversion of the carboxylic acid groups to carboxylates leads to stable aqueous solutions due to charge repulsion between the individual nanostructures.
Figure 2.6. TGA traces of the SWCNTs and PCP-SWCNT-1 (functionalized 1 time) in an Ar atmosphere at a 10 °C/min scan rate from RT to 700 °C.
Figure 2.7. TGA traces of the US-tubes, PCP-US-tubes-1 (functionalized 1 time), PCP-US-tubes-2 (functionalized 2 times), PCP-US-tubes-3 (functionalized 3 times), PCP-US-tubes-4 (functionalized 4 times) in an Ar atmosphere at a 10 °C/min scan rate, from RT to 700 °C, and first derivative of the PCP-US-tubes-4 trace.
Figure 2.8. TGA traces for the GNRs and PCP-GNRs 1-4 (PCP-GNRs functionalized repeatedly 1-4 times) in an Ar atmosphere at a 10 °C/min scan rate from 100 to 650 °C.

Raman spectra of the PCP-SWCNTs (Figure 2.9) showed changes compared to the spectrum of the original SWCNT sample. The intensity of the disorder mode (1314 cm$^{-1}$) for the PCP-SWCNTs is increased due to a greater number of sp$^3$ carbons, compared to SWCNTs, which is indicative of covalent bond formation. Raman spectra of the PCP-SWCNTs performed before and after TGA (Figure 2.9) showed that the disorder mode (1314 cm$^{-1}$) decreases in intensity after TGA, which is indicative of a sp$^2$ honey-comb structure restoration. The intensities of the radial breathing mode (161 cm$^{-1}$) is less for the PCP-SWCNTs and PCP-US-tubes comparing to SWCNTs and US-tubes (Figure 2.9 and Figure 2.10), thereby indicating the presence of functional groups. The presence of both tangential (1585 cm$^{-1}$)
and radial breathing modes of (161 cm\(^{-1}\)) of PCP-SWCNTs and PCP-US-tubes indicate the preservation of the tubular structures. Raman spectra of the GNRs before and after the functionalization does not show changes because the surface is less populated with functional groups, as also seen from the TGA data (Figure 2.8), and because of its multi-layer structure (40–50 layers) where just the outer layer is exposed for functionalization.

![Raman spectra](image)

**Figure 2.9.** Raman spectra (633 nm excitation) of SWCNT, PCP-SWCNT and TGA-PCP-SWCNT-1 from Figure 2.6 after heating to >700 °C in the TGA experiment. Increase in intensity of the disorder mode (1314 cm\(^{-1}\)) for the PCP-SWCNT spectrum indicates increased number of sp\(^3\) carbons then comparing to SWCNTs, hence formation of covalent bond. TGA-PCP-SWCNT-1 spectrum as a result of decomposition of covalently-attached moieties and restoration of the sp\(^2\) structure of the initial material.

XPS showed the difference between the GNR, SWCNT, US-tube starting materials and their functionalized derivatives. The peak at 284–285 eV in Figure 2.11 corresponds to C1s of the GNRs, SWCNTs and US-tubes\textsuperscript{38} and to the aromatic ring C1s on the attached phenyl groups. Peaks at 288 eV for the PCP-US-tubes and PCP-SWCNTs are indicative of the presence of carboxylic acid groups on the surface of PCP-US-tubes-4 and PCP-SWCNTs-4.\textsuperscript{38} A carboxylic acid peak for the PCP-GNRs is not observed for the same reason because of the lack of functionalization, as discussed above.
High zeta potential values for aqueous colloidal solutions of the PCP-GNRs (−55 mV), PCP-SWCNTs (−51 mV), PCP-US-tubes (−61 mV) are indicative of high solution stability according to the American Society for Testing and Materials (ASTM), which defines colloids with zeta potentials higher than 40 mV (negative or positive) to have good stability. A negative value for the zeta potential
corresponds to negatively-charged species on the surface of the carbonous material, which are carboxylates in this case. The stability of the colloid solutions at different ionic strengths and pH values was also examined. PCP-GNRs were found to be stable in 0.05 M NaCl and 0.05 M NaOH aqueous solutions. Addition of HCl to the PCP-GNR solution protonates the carboxylate groups to reduce the overall negative charge on the surface of the nanostructure which leads to lower aqueous stability. An aqueous solution of the PCP-GNRs with 0.001 M HCl produced a zeta potential increase to −13.7 mV and a solution of 0.05 M HCl to +3.83 mV, which is additional evidence for the present of carboxylate groups on the surface of the PCP-GNR. PCP-SWCNTs and PCP-US-tubes continue to have good stability under the same conditions as for the PCP-GNRs, except that the PCP-US-tubes in 0.05 M NaCl showed somewhat lower stability with a zeta potential of −34.5 mV.

2.4. Conclusions

In summary, the present chapter reports a new, rapid (1–2 h) and universal procedure to prepare highly water soluble carbon nanostructures that include full-length SWCNTs, ultra-short SWCNTs, and newly-available GNRs. For the first time, this new process employs an environmentally-friendly procedure that utilizes a direct, multi-step and scalable process for derivatizing carbon nanostructures. The procedure has already produced the most water-soluble carbon nanotube material known for ultra-short SWCNTs (50 mg/ml), and by modifying the substituted aryl diazonium salt for non-aqueous solvents, low-defect-site GNR materials might also be realized for electronic material applications.
Chapter 3

Enhanced MRI relaxivity of aquated Gd$^{3+}$ ions by carboxyphenylated water-dispersed graphene nanoribbons

3.1. Introduction

Since the discovery of graphene by Geim and Novoselov in 2004, extensive research has been performed in diverse fields of science, engineering, and medicine to take advantage of its unique properties.$^{40-43}$ Properties such as excellent electrical conductivity, thermal stability, mechanical strength, and other unusual chemical and optical properties have made graphene an extensively studied...
material for composites, transistors, biosensors, nanomedicines, and many other types of materials requiring the exceptional properties of graphene and graphene-based materials.

Magnetic resonance imaging (MRI) is one of the most powerful imaging tools in the clinic for non-invasive diagnostics. Many of its capabilities depend on the use of chemical contrast agents (CAs), which are employed to enhance local signal intensity to improve diagnostic confidence.

Image contrast in MRI arises largely from differences in water-proton spin-lattice relaxation time ($T_1$) and spin-spin relaxation times ($T_2$). Thus, chemical CAs are categorized as either $T_1$ or $T_2$ agents based on the efficiency of changing $T_1$ or $T_2$ in the observable water pool. $T_1$ CAs are mainly Gd$^{3+}$-based and provide brighter images, whereas $T_2$ CAs produce darker images and are usually superparamagnetic iron oxide (SPIO) nanoparticles. Relaxivity ($r_{1,2}$) is the parameter of CAs which defines their efficacy and is the relaxation rate enhancement in 1 mM$^{-1}$ s$^{-1}$ of the CA.

There has been extensive effort to develop new CAs with increased relaxivities using small Gd$^{3+}$-ion chelates, dendrimers, polymers, micelles, and various nanostructures. In recent years, a great diversity of nanostructure-based CAs have been developed which exhibit high relaxivity. In particular, honeycomb carbon nanostructures such as gadofullerenes, gadonanotubes, and gadographene have shown the highest relaxivity values for the Gd$^{3+}$ ion. In this work, a new type of carbon nanoconstructed-based CA is reported, with Gd$^{3+}$
ions conjugated to covalently functionalized highly water-dispersed graphene nanoribbons (Gd/GNRs) to produce greatly enhanced $r_1$ and $r_2$ values ($70 \pm 6$ mM$^{-1}$ s$^{-1}$ and $108 \pm 9$ mM$^{-1}$ s$^{-1}$, respectively) at 1.41 T and 37 °C. These values are up to 16 times and 21 times higher for $r_1$ and $r_2$ respectively, when compared to clinically available Magnevist® CA with $r_1 \approx 4$ mM$^{-1}$ s$^{-1}$ and $r_2 \approx 5$ mM$^{-1}$ s$^{-1}$ at 1.5 T.$^{50}$ Unlike the recently reported and related gadographene CA,$^{58}$ the present Gd/GNR CAs do not require a surfactant to suspend them in water and biological media.

### 3.2. Materials and Methods

#### 3.2.1. Preparation and characterization of the Gd/GNRs.

Synthesis of the GNRs with a width of 125–280 nm, thickness of 7–15 nm, and length of up to 20 μm was achieved by chemically splitting multi-walled carbon nanotubes (MWCNTs) using K/Na alloy,$^{12}$ unlike the preparation of GNRs that uses KMnO$_4$.$^{4,59}$ The GNRs were then repetitively derivatized with $p$-carboxyphenyldiazonium salt to make them highly water dispersed (4.7 mg/mL) and to provide carboxylic acid groups for Gd$^{3+}$ coordination.$^6$ Next, an aqueous solution of GdCl$_3$ (30 mg in 3 mL) was added to an aqueous dispersion (10 mL) of GNRs (10 mg) and the mixture was sonicated in a bath for 15 min. The mixture was then vacuum filtered using an Anapore™ inorganic membrane disc with a pore size of 0.02 μm (Anodisc™) and thoroughly washed with DI water multiple times until no Gd$^{3+}$ ions were present in the filtrate (confirmed by ICP-OES). The black solid collected on the filter (Gd/GNRs) was then characterized using transmission
electron microscopy (TEM) equipped with electron dispersion spectroscopy (EDS), X-ray photoelectron spectroscopy (XPS), inductively-coupled plasma optical emission spectrometry (ICP-OES), a benchtop relaxometer (Bruker Minispec) operating at 1.41 T and 37 °C with a 5 mm probe, a clinical 1.5 T MRI scanner (Philips Achieva), and a fast field cycling nuclear magnetic resonance (NMR) spectrometer FFC-200 (Stellar s. r. l., Mede, Italy).

3.2.2. MRI acquisition parameters.

The $T_1$ relaxation times of the samples were measured using an inversion recovery prepared spin echo sequence with the following acquisition parameters: acquisition voxel size: 1.25 $\times$ 1.25 $\times$ 5 mm$^3$; TR/TE ms: 15000 ms / 8.8 ms; scan time: 16 min. The experiment was repeated at various inversion times (TIs): 100ms, 300ms, 500ms, 1000ms, 1500ms, 2500ms, 3500ms, and 4500ms. The $T_2$ relaxation times of the samples were measured using a multi-echo spin echo sequence with the following acquisition parameters: acquisition voxel size: 1.25 $\times$ 1.25 $\times$ 5 mm$^3$; TR/TE ms: 15000 ms / 25 ms; 32 echos were measured with echo spacing of 25ms; Scan time: 16 min.
3.3. Results and Discussion

To better understand the nature and presence of the Gd$^{3+}$ ions on the surface of the GNRs (Figure 3.1), TEM equipped with an EDS was used. The top row of Figure 3.2 shows TEM images of the Gd/GNRs (Figure 3.2 a) and GNRs (Figure 3.2 b) with corresponding EDS mappings (mapping areas designated by dotted white lines) on the bottom row for Gd$^{3+}$ and Cu$^{2+}$ of the Gd/GNR sample (Figure 3.2 c) and the GNR sample (Figure 3.2 d). Cu$^{2+}$ and Gd$^{3+}$-ion mapping are shown together due to the overlap of the spectral peaks of these two elements; the trace of the Cu$^{2+}$-ion signal is due to the copper grid TEM sample holder. From the EDS images it is clear that the Gd/GNR sample surface is covered with Gd$^{3+}$ ions (white spots) (Figure 3.2 c), whereas the GNR sample is not (Figure 3.2 d). Coverage of the GNR surface with Gd$^{3+}$ ions was produced by coordination of the ions to the carboxylate groups of the GNRs. The EDS spectrum (Figure 3.2e) of the Gd/GNRs also shows the presence of...
Gd$^{3+}$ ions ($\approx$1 keV and $\approx$ 8 keV) with traces of Cl$^-$ ion ($\approx$3 keV) coming from the GdCl$_3$ used to prepare the Gd/GNRs.

Figure 3.2. TEM images of (a) Gd/GNRs and (b) GNRs. Electron dispersion spectroscopy (EDS)-TEM mapping for Gd$^{3+}$ and Cu$^{2+}$ of (c) Gd/GNRs and (d) GNRs (mapping areas designated by dotted white lines), and (e) EDS spectra of the Gd/GNRs and GNRs (scale bars = 600 nm).
XPS data (Figure 3.3) also provides evidence for the presence of Gd$^{3+}$ on the surface of Gd/GNR sample. The photoelectron peak corresponding to the Gd$^{3+}$ ion 4d level was observed at $\approx 141$ eV for a Gd/GNR sample, whereas for a GNR sample no peak was observed in the region. Peaks for Si at 153 eV (Si2s) and 102 eV (Si2p) are due to contamination from the Pyrex ampule during synthesis of the GNRs from MWCNTs. Na$^+$ ion ($\approx 1071$ and 496 eV) was introduced to the sample during the functionalization step.\(^6\)

![Figure 3.3. XPS data for the GNR and Gd/GNR samples.](image)

The amount of Gd$^{3+}$ in a solid Gd/GNR sample was determined to be $1.5 \pm 0.4\% \text{ w/w}$ by ICP-OES. For the determination, a known amount of Gd/GNRs (0.2–0.5 mg) was carefully heated in 36% w/w HClO$_3$ (1–2 mL) to completely remove any
remaining carbonaceous material and then reconstituted in 5 mL 2% HNO₃ for ICP-OES analysis.

Relaxation times $T₁$ and $T₂$ were measured using a Bruker Minispec operating at 1.41 T and 37 °C. Gadolinium concentrations were determined using ICP-OES and a linear relation between relaxation rate and concentration was found (Figure 3.4) with slopes of $70 \pm 6$ mM$^{-1}$ s$^{-1}$ for $r₁$ and $108 \pm 9$ mM$^{-1}$ s$^{-1}$ for $r₂$ ($n = 4$). $T₁$ was also measured as a function of magnetic field strength using a Stelar Spin Master field cycling spectrometer (Mede, Italy). Samples were contained in 10 mm tubes at 25 °C.

Figure 3.4. Plots of [Gd$^{3+}$] vs 1/$T₁$ and 1/$T₂$ for the Gd/GNR sample at 1.41 T and 37 °C. The slopes of the least-squared fitted red lines represent the relaxivity ($r₁$, $r₂$) values per Gd$^{3+}$ ion.

The effectiveness of the Gd/GNRs as a $T₁$ and $T₂$ CA was assessed via a 1.5 T MRI clinical scanner. From the $T₁$-weighted phantom images (Figure 3.5a) obtained at different inversion times (TI) for the same amount (0.225 mg/mL) of aqueous
dispersion of GNRs, Gd/GNRs (≈ 1.7% Gd w/w) and H₂O samples it is clear that there is great contrast enhancement for the Gd/GNR sample, when compared to GNRs and H₂O. Similarly, from the \( T_2 \)-weighted phantom images (Figure 3.5b) acquired at different echo times (TE) for the same samples, there is clearly observable contrast enhancement for the Gd/GNR sample when compared to GNRs and H₂O. No contrast enhancement for the GNRs alone when compared to H₂O is presented in order to show that there is no relaxivity contribution due to possible trace metal impurities. The images presented in Figure 3.5 show the efficacy of the Gd/GNRs as a CA for \( T_1 \)- and \( T_2 \)-weighted imaging.

\[ \text{Figure 3.5. (a) } T_1 \text{-weighted MRI inversion recovery phantom images acquired at different inversion times (TI) for the GNR, Gd/GNR samples in aqueous dispersions and H}_2\text{O at 1.5 T. (b) } T_2 \text{-weighted MRI spin-echo phantom images acquired at different echo times (TE) for the GNR, Gd/GNR samples in aqueous dispersions and H}_2\text{O at 1.5 T.} \]
To better understand the binding nature of Gd$^{3+}$ to the GNRs, strong acid (1 M HCl), strong base (1 M NaOH), and DI water washings were performed on the Gd/GNR sample. To determine the results for a known amount of Gd/GNRs, samples were dispersed in 1 M HCl, 1 M NaOH, and deionized water and the dispersions were then sonicated for 10 min before filtration through 0.22 μm glass syringe filters. Filtrates were analyzed via ICP-OES for the presence of Gd$^{3+}$. It was found that 1 M HCl removes the Gd$^{3+}$ ions from the surface of the GNRs. Repeating this procedure with 1 M NaOH or DI water did not remove any detectable Gd$^{3+}$. These results support the assumption that the Gd$^{3+}$ ions are coordinated to carboxylate groups present on the surface of the functionalized GNRs, since only protonation of these groups by acid removes the Gd$^{3+}$ ions.

To check for the physiological stability of the Gd$^{3+}$ ions in the Gd/GNRs, 1× phosphate buffered saline (PBS) and fetal bovine serum (FBS) in vitro challenges were performed. In two separate experiments, a Gd/GNR sample was challenged for 24 h at 37 °C in 1× PBS and FBS. The resulting dispersions were filtered through a 0.22 μm glass syringe filter and the filtrates were analysed for Gd$^{3+}$ content with ICP-OES. 1 × PBS challenge did not produce any Gd$^{3+}$ ion loss, while the FBS challenge resulted in a loss of ca. 50 ± 10% (n = 3) of the Gd$^{3+}$ ions from multiple samples functionalized at different times. After the first FBS challenge, the same sample did not lose additional Gd$^{3+}$ ions with subsequent challenges. In the case of PBS, no Gd$^{3+}$ loss was observed, since no strongly chelating agents or ions capable of replacing Gd$^{3+}$ are present in PBS. For FBS, the proteins present are good chelating agents and are apparently capable of removing loosely bound Gd$^{3+}$ ions from the
surface of the Gd/GNRs. Neither of the challenges resulted in measurable changes in $r_1$ or $r_2$ for the coordinated to GNRs Gd$^{3+}$ ion. Thus, due to the material nature of the Gd/GNRs, there appear to be different kinds of coordination sites present, and after the first FBS challenge the same sample becomes stable to subsequent FBS challenges at physiological pH and temperature.

The relaxation rate constants for the Gd/GNRs ([Gd] = 0.0345 mM) sample (Figure 3.6) are large and demonstrate that the Gd$^{3+}$ ion first coordination sphere waters exchange rapidly with bulk water. The low field relaxation rate is essentially constant, but with increasing field there is a decrease followed by an increase and a maximum, which is not observed in the present experiments. This relaxation dispersion profile is characteristic of a slowly rotating Gd$^{3+}$-ion complex. The increase in relaxation rates at high field derives from the magnetic field dependence of the electron-spin relaxation rate constant which makes the effective correlation time for the electron-nuclear coupling a function of magnetic field. Thus, the nuclear spin-relaxation properties are decoupled from the rotational and exchange dynamics of the Gd$^{3+}$ complex throughout the magnetic field range studied here. The dominance of electron spin relaxation is the rule in slowly rotating Gd$^{3+}$ complexes. Contributions from non-metal radicals, which are common adjuncts of polynuclear aromatic preparations would generally induce nuclear spin relaxation by outer sphere or translational diffusive motions of the water in the vicinity of the radical. The shape of the relaxation dispersion profile from this class of dynamics is characteristically a constant relaxation rate at low field and a decrease in relaxation rate constant at high field without the dramatic increase observed in Figure 3.6. It is
also possible that magnetic domains may form in particulate systems that, in some instances, may present a relaxation dispersion profile similar to that shown in Figure 3.6.\textsuperscript{61} However, we have no evidence that such organization occurs in the present preparations.

![Graph](image)

**Figure 3.6.** The spin-lattice-relaxation normalized to Gd\textsuperscript{3+} concentration as a function of magnetic field strength reported as the \( ^1\text{H} \) Larmor frequency at 0.01–20 MHz and 25 °C for water protons in an aqueous suspension of the Gd/GNRs.

The above results demonstrate that Gd\textsuperscript{3+} ions in Gd/GNRs are coordinated to the carboxylate groups of the GNRs. The number of the Gd\textsuperscript{3+}-ion coordination sites occupied by the carboxylate groups is unclear, even though the total coordination number for individual Gd\textsuperscript{3+} ions is normally 8 or 9.\textsuperscript{62,63} It is known that the \( r_1 \) relaxivity of a Gd\textsuperscript{3+}-ion CA depends on many parameters, but the most dominant
factors are the tumbling time, the water-exchange rate, and the number of available sites for water coordination. In our case, the large \( r_1 \) and \( r_2 \) relaxivity values are most likely due to a larger number of water coordination sites on the Gd\(^{3+}\) ion.

### 3.4. Conclusions

In conclusion, it has been shown that surfactant-free carboxyphenylated water-dispersed graphene nanoribbons are capable of increasing \( r_1 \) to 70 ± 6 mM\(^{-1}\) s\(^{-1}\) and \( r_2 \) to 108 ± 9 mM\(^{-1}\) s\(^{-1}\) for Gd\(^{3+}\) ions coordinated to the carboxylate groups. These values are 16 and 21 times greater when compared to current clinically-available Gd\(^{3+}\)-based \( T_1 \) CAs, and about the same as found for gadographene suspended with surfactant. Because the Gd/GNRs are lipophilic and stable to Gd\(^{3+}\)-ion loss under physiological conditions (after one FBS challenge), they possibly can be safely used to internally label cells for MRI tracking \textit{in vivo}, similar to other Gd\(^{3+}\)-ion containing carbon nanostructures such as the gadofullerenes or the gadonanotubes. Of the four gadocarbon nanostructure labeling agents (gadofullerenes, gadonanotubes, gadographene, and Gd/GNRs), the much greater availability of the last three suggests that they might become the preferred cell labeling agents. Of these three, the covalently-functionalized Gd/GNRs could have advantages over the surfactant-wrapped nanostructures, especially if the surfactant wrapping is unstable to cell membrane transport during the cell labeling process. We are presently exploring this possibility.
Chapter 4

Hierarchically Structured Magnetic Nanoconstructs with Enhanced Relaxivity and Cooperative Tumor Accumulation

4.1. Introduction

Iron oxide nanoparticles (IOs) exhibit interesting multi-functional properties that could be used in a variety of biomedical applications. However, a major problem preventing to their full utilization is that these properties manifest themselves over different, non-overlapping, size intervals. In magnetic resonance

imaging (MRI), IOs with a diameter smaller than 100 nm induce significant shortening in transversal relaxation times, $T_2$. On the other hand, only micron and sub-micron sized IOs have been efficiently manipulated in vivo by remote static magnetic fields. In therapeutic applications, 20–50 nm IOs have been used as nanoheaters in that, under alternating magnetic fields, they can generate significant doses of thermal energy for ablating the surrounding malignant tissue, sensitizing cells in adjuvant therapies, and triggering the release of active molecules. Moreover, only IOs smaller than ≈20 nm can be fully degraded and metabolized over a few days. A novel strategy is needed to effectively decouple the IO functions from the particle size in order to fully capitalize on their multiple functionalities.

MRI is considered a powerful tool in tumor imaging because of its non-invasiveness and high spatial resolution. However, clinically available products, such as Feridex, often exhibit relatively low transversal relaxivities ($r_2$), typically of about 100 mm$^{-1}$ s$^{-1}$. Different strategies have been explored to improve the MRI performance of IO-based contrast agents, including: the modulation of their size, shape and surface properties and the use of metal alloys. It is well documented that $r_2$ can be enhanced by increasing the size of the magnetic core, using cubically shaped nanoparticles, and decorating the particle surface with molecules and polymers. Also, the inclusion of atoms such as Co, Mn, Ni in the magnetic core has improved the relaxometric response. However, none of the listed approaches can successfully combine the multiple functionalities of the IOs. Recently, it has been realized that IO clusters can also provide higher relaxivities when compared to individual particles. However, such an enhancement is strictly depending on the
spatial organization and level of hydration of IOs. Moreover, controlling and preserving the state of aggregation of the USPIO clusters in vivo, upon systemic injection, without compromising their biodistribution are major, unsolved challenges.

Contrast enhancement and therapeutic efficacy are directly related to the amount of IOs accumulated at the diseased site. Although IOs with ‘stealth’ coatings tend to circulate longer, thus offering a higher probability of passive accumulation within the tumor mass, the amount of IOs reaching the tumor site can be much smaller than 1% of the injected dose per gram tissue (% ID/g). Remote guidance via external magnetic fields has been proposed as a way to attract more IOs within the target tissue. However, magnetic forces scale proportionally to the particle volume and drop rapidly as the IO diameter decreases, being negligibly small, as compared to hydrodynamic and colloidal interactions, for particles of a few tens of nanometers. Also, the magnetic force reduces with the distance from the magnet and the penetration depth. Therefore, not surprisingly, magnetic targeting has provided some success only for particles larger than 100 nm and injected doses of the order of 10 mg of Fe kg$^{-1}$ of animal.

Here, to fully capitalize on the intrinsic multi-functional capabilities of IOs, a novel approach is proposed to boost the relaxivity $r_2$, enhance tumor accumulation by remote magnetic guidance, and, possibly, enable hyperthermia treatments upon systemic injection. This is obtained by dispersing multiple clusters of small USPIOs within the mesoporous silicon particles (SiMPs), thus leading to the formation of
multiscale, hierarchically organized magnetic nanoconstructs. Commercially available USPIOs are used with a nominal magnetic core diameter of 5 nm. The SiMPs have been rationally designed to deposit within the tumor vasculature by relying on the balance between hydrodynamic dislodging forces and interfacial adhesion interactions with the blood vessel walls.\textsuperscript{100–103} In this chapter, first, the USPIO loading efficiency and the stability of the resulting nanoconstructs have been analyzed, under physiologically relevant conditions. Secondly, the relaxometric and magnetic guidance response of the nanoconstructs have been characterized \textit{in vitro}. Finally, for the SiMPs which have been extensively tested \textit{in vivo},\textsuperscript{102–104} the MRI and magnetic guidance performance have been demonstrated in a melanoma mouse model using a 3T MRI clinical scanner.

\section*{4.2. Materials and Methods}

\subsection*{4.2.1. Purification and characterization of the superparamagnetic iron oxide nanoparticles (USPIOs).}

For the hydrophilic USPIOs loaded into the SiMPs, the samples as provided by the vendors presented several aggregates, and the following procedure was performed in order to purify the original solution and select the USPIOs with the higher stability in solution. Upon sonication (~ 15 min – Branson Ultrasonic Cleaner), the samples were centrifuged (6 minutes, at 12,000 rpm) and the supernatants were collected. This step was repeated twice. The resulting supernatant was used for all the experiments.
For assessing the iron content in the purified solution, the colloidal suspension was digested in 70 % nitric acid (Sigma-Aldrich, ≥ 99.999% purity, trace metal grade) on a thermoplate at 110 °C. The resulting solution was re-suspended in 5 ml of 2 % nitric acid and filtered (0.22 µm pore size). Finally, the sample was analyzed via Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) for elemental Fe content.

The size of the USPIO magnetic core was measured via Transmission Electron Microscopy (JEM-2100F TEM, JEOL Ltd.). Purified samples were diluted in DI water 10 times and 10 µL of the solution was deposited onto the surface of a TEM grid (Ted Pella, Inc., Formvar/Carbon 400 mesh, Copper, approx. grid hole size: 42µm) and left to dry for 1h.

**4.2.2. Fabrication, loading and Physico-chemical characterization of the USPIO-loaded Discoidal Mesoporous Silicon Particles (SiMPs).**

Discoidal porous silicon particles were fabricated by using previously reported protocols. The fabrication process consists of three major steps: formation of porous silicon film, photolithographic patterning of particles, and Reactive Ion Etch (RIE). The porous structure was tailored by electrochemical etching while the particle sizes were precisely defined by photolithography. Since the porous structure and the particle size are controlled independently, a wide range of sizes, shapes and pore morphologies can be obtained using such an approach. In this work, particles with 1,000 nm in diameter and 40 nm in thickness were used. These particles have a mean pore size of 40 nm and a porosity of about
60%. The fabrication process is briefly described as follows: starting with heavily doped P-type (100) wafer with resistivity of 0.005 ohm·cm as the substrate (Silicon Quest, Inc, Santa Clara, CA), the wafer was assembled on a home-made anodizing cell with the polished surface immersing in 1:3 HF (49 %):ethanol solution. An etching current of 6 mA/cm² was applied for 125 sec to generate 400 nm porous silicon film. Then a high electrical current with current density ~76 mA/cm² was applied for 8 sec to form the unstable release layer. An 80 nm low-temperature oxide (LTO) was deposited on the porous silicon film in a LPCVD furnace. A standard photolithography process was used to pattern the 1,000 nm circles on the film using a contact aligner (SUSS MA6 mask aligner) and NR9-500P photoresist (Futurrex Franklin, NJ, USA). The pattern was transferred into the porous silicon film by RIE in CF₄ plasma (Plasmatherm BatchTop, 15 sccm CF₄, 100 mTorr, 200 W RF). After striping the LTO, the porous silicon disk arrays were released in isopropanol solution by ultrasound for 1 minute. The SEM imaging of the particles was performed using a ZEISS NEON 40 Scanning Electron Microscope (SEM) at 5 kV and 3-5 mm working distance using an In-lens detector. (Figure 4.1) The volume, size and concentration of particles were characterized by a Multisizer 4 Coulter Counter (Beckman Coulter). Their surface charge was measured in a phosphate buffer at pH 7.4 using a ZetaPALS Zeta Potential Analyzer (Brookhaven Instruments Corporation; Holtsville, NY).
As per the loading of USPIOs into SiMPs, the stock solutions of USPIOs as purchased was first purified to select particles with higher aqueous stability, following the sequential steps described above. Then, SiMPs were lyophilized to dryness for 8 hours and $2 \times 10^8$ SiMPs were exposed to 100 µL of the purified USPIO solution. The resulting suspension was sonicated (30 W bath sonicator) for 1 min and centrifuged for 10 min at 4,000 rpm. The supernatant was decanted and the precipitate washed twice with 200 µL deionized water to remove any free USPIOs from the SiMP surface. The resulting assembly was resuspended in 200 µL of deionized water and characterized using HR-TEM (JEOL 2000 FX) equipped with an energy-dispersive spectrometer (EDS) and ICP-OES. For TEM and EDS, samples were diluted (10 X) with DI water and 10 µL of the nanoconstructs solution was dropped onto a surface of TEM grid (Ted Pella, inc., Formvar/Carbon 400 mesh, Copper, approx. grid hole size: 42 µm). The USPIO loading was quantified by dissolving the loaded SiMPs in 0.1 M NaOH (99.99 % trace metals basis) overnight with further digestion in 70 % HNO₃. The samples were heated to dryness and were reconstituted in 2 % HNO₃. The concentration of Fe was measured using ICP-OES.
4.2.3. Magnetic Force Microscopy characterization of the nanoconstructs.

5 nm USPIOs-loaded SiMPs and SiMPs alone were analyzed via Magnetic Force Microscopy (MFM) using a MultiMode 8 Atomic Force Microscope (Bruker). The cantilever used was a commercial MESP (Bruker) Antimony (n) doped Si probe with a resistivity of 0.01 – 0.025 Ωcm, a rectangular geometry, a nominal thickness of 2.75 μm, and a front side coated by magnetic Co/Cr. The nominal resonant frequency and spring constant were 75 KHz and 2.8 N/m, respectively. Sample drops from the nanoconstructs were deposited over a mica sheet and analyzed when dried.

4.2.4. Molecular dynamics simulation of self-diffusion coefficient for water molecules in porous structures.

In order to estimate the self-diffusion coefficient of nano-confined water within silica nanopores at different USPIOs concentrations, a pore with a diameter D = 8 nm and a computational box with size 11.3 x 11.06 x 4.32 nm³ were considered. Periodic boundary conditions are applied along all directions mimicking an infinite array of pores with infinite length, and each USPIO (with fixed radius R = 1 nm) is initially inserted in the vicinity of the pore surface. Water solvation follows after the positioning of a given number of nanoparticles. Three configurations were studied with low (two USPIOs in the computational box), medium (eight USPIOs in the computational box) and high (sixteen USPIOs in the computational box) USPIO loading.
Silica pore preparation. The alpha-quartz (SiO$_2$) unit cell (left-hand side of Figure 4.2) was firstly considered.$^{106}$ The latter unit cell is replicated along all Cartesian coordinates, such that a fully periodic brick with size 11.3 x 11.06 x 4.32 nm$^3$ is constructed (middle picture in Figure 4.2). Finally, a pore in the above brick is obtained by removing atoms whose coordinates are contained within a given distance (pore radius) from the center. All rendering pictures of the paper were made by UCSF Chimera.$^{107}$

Figure 4.2. Preparation of a silica pore. From the left hand side: Alpha-quartz (SiO$_2$) unit cell, periodic brick and the silica pore.

Upon the creation of the pore, all silicon atoms located along the ‘cut surface’, with only one bonded oxygen, are removed. At the same time, one hydrogen atom is attached to all oxygen atoms which are missing one bonded silicon (Figure 4.3). This is achieved imposing that the angle formed by silicon, oxygen and hydrogen is 128.8 degrees (elevation), with random azimuth angle.
Figure 4.3. Treatment of the pore walls. A hydrogen atom is attached to the surface oxygens that are missing one bond with silicon (left column). Silanol groups (Si-O-H) are formed at the pore surface (right column).

**Force fields for silica.** The above silica structure has been modeled by means of the open-source molecular dynamics (MD) simulation package GRoningen MACHine for Chemical Simulations (GROMACS). More specifically, two harmonic terms are used to describe the silicon-oxygen and oxygen-hydrogen interactions. A bond stretching potential between two bonded atoms $i$ and $j$ at a certain distance (around the equilibrium distance), and a bending angle potential (between the two pairs of bonded atoms $(i,j)$ and $(j,k)$) are considered as follows (Equation 4.1):

$$V_{Si}(r_i, \theta_{ijk}) = \frac{1}{2} k_{ij}^b (r_{ij} - r_{ij}^0)^2 + \frac{1}{2} k_{ijk}^\theta (\theta_{ijk} - \theta_{ijk}^0)^2$$

**Equation 4.1.** The values for each parameters reported in the following Table 4.1.
<table>
<thead>
<tr>
<th>Interaction</th>
<th>$k_{ij}^b$ (kJ mol$^{-1}$ nm$^{-2}$)</th>
<th>$k_{ijk}^\vartheta$ (kJ mol$^{-1}$ rad$^{-2}$)</th>
<th>$r_{ij}^0$ (nm)</th>
<th>$\theta_{ijk}^0$ (deg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Si - O(Si)</td>
<td>126356.8</td>
<td>-</td>
<td>0.161</td>
<td>-</td>
</tr>
<tr>
<td>Si - O(H)</td>
<td>135980.0</td>
<td>-</td>
<td>0.161</td>
<td>-</td>
</tr>
<tr>
<td>H - O(H)</td>
<td>236814.4</td>
<td>-</td>
<td>0.096</td>
<td>-</td>
</tr>
<tr>
<td>O - Si - O</td>
<td>-</td>
<td>125.520</td>
<td>-</td>
<td>109.800</td>
</tr>
<tr>
<td>O - Si - O(H)</td>
<td>-</td>
<td>133.888</td>
<td>-</td>
<td>109.800</td>
</tr>
<tr>
<td>Si - O - Si</td>
<td>-</td>
<td>142.256</td>
<td>-</td>
<td>109.800</td>
</tr>
<tr>
<td>Si - O(H) - Si</td>
<td>-</td>
<td>142.256</td>
<td>-</td>
<td>143.600</td>
</tr>
<tr>
<td>Si - O(H) - H</td>
<td>-</td>
<td>142.256</td>
<td>-</td>
<td>128.800</td>
</tr>
<tr>
<td>O(H) - Si - O(H)</td>
<td>-</td>
<td>125.520</td>
<td>-</td>
<td>109.800</td>
</tr>
</tbody>
</table>

Table 4.1. Parameters for silica bonded interactions.$^{109}$

where ‘Si’ are bulk silicon atoms, ‘O(Si)’ are bulk oxygen atoms, ‘O(H)’ are superficial oxygen atoms (which belong to a sylanol group) and ‘H’ are hydrogen atoms.

Moreover, the non-bonded interactions include a Lennard-Jones term as follows (Equation 4.2):

$$V(r_{ij}) = 4\varepsilon_{ij}\left[\left(\frac{\sigma_{ij}}{r_{ij}}\right)^{12} + \left(\frac{\sigma_{ij}}{r_{ij}}\right)^{6}\right]$$

**Equation 4.2.**
where parametrization is consistent with the following Lorentz-Berthelot combination rule (Equation 4.3):\textsuperscript{110}

\[
\begin{align*}
\sigma_{ij} &= \frac{1}{2} (\sigma_i + \sigma_j) \\
\varepsilon_{ij} &= (\varepsilon_i + \varepsilon_j)^{1/2}
\end{align*}
\]

Equation 4.3.

Moreover, a Coulomb term is considered as well (Equation 4.4):

\[
V(r_{ij}) = \frac{1}{4\pi\varepsilon_0} \frac{q_i q_j}{r_{ij}}
\]

Equation 4.4. $\varepsilon_0$ is the permittivity in vacuum, and $q_i$ is the partial charge of the atom $i$.

Parameters for non-bonded interactions are reported in the following Table 4.2:\textsuperscript{110}
<table>
<thead>
<tr>
<th>Atom</th>
<th>$\sigma_{ii}$ (nm)</th>
<th>$\epsilon_{ii}$ (kJ mol$^{-1}$)</th>
<th>$q_i$ (e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Si (1 silanol group)</td>
<td>0.3154</td>
<td>0.6487</td>
<td>0.31</td>
</tr>
<tr>
<td>Si (2 silanol groups)</td>
<td>0.3154</td>
<td>0.6487</td>
<td>0.62</td>
</tr>
<tr>
<td>Si (3 silanol groups)</td>
<td>0.3154</td>
<td>0.6487</td>
<td>0.93</td>
</tr>
<tr>
<td>O (surface)</td>
<td>0.3795</td>
<td>0.5336</td>
<td>-0.71</td>
</tr>
<tr>
<td>Si (bulk)</td>
<td>0.3154</td>
<td>0.6487</td>
<td>0</td>
</tr>
<tr>
<td>O (bulk)</td>
<td>0.3795</td>
<td>0.5336</td>
<td>0</td>
</tr>
<tr>
<td>H</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.40</td>
</tr>
</tbody>
</table>

**Table 4.2. Parameters for silica non-bonded interactions.**

Lennard-Jones potentials were treated with a twin-range cut-off and 1.5 nm cut-off distance, whereas for the electrostatic interactions, a Particle Mesh Ewald (PME) was used with 1.5 nm real-space cut-off, a 0.12 nm reciprocal space gridding, and splines of order 4 with $10^{-5}$ tolerance.

**Water model and corresponding force field parameters.** The SPC/E model is used for describing water, consisting in intramolecular harmonic stretching and bending between the hydrogen and oxygen atoms as (Equation 4.5):

$$V_w(r_{ij}, \theta_{ijk}) = \frac{1}{2} k_{ij}^W (r_{ij} - r_{ij}^W)^2 + \frac{1}{2} k_{ijk}^{\theta W} (\theta_{ijk} - \theta_{ijk}^{\theta W})^2$$

**Equation 4.5.**
Non bonded interactions include a Lennard-Jones term between oxygen atoms as follows (Equation 4.6):

\[
V(r_{ij}) = 4\varepsilon_{OO} \left[ \left( \frac{\sigma_{OO}}{r_{ij}} \right)^{12} + \left( \frac{\sigma_{OO}}{r_{ij}} \right)^{6} \right]
\]

**Equation 4.6.**

where \( \sigma_{oo} = 0.3166 \,[\text{nm}] \) and \( \varepsilon_{oo} = 0.6502 \,[\text{kJ mol}^{-1}] \), along with a Coulomb potential (Equation 4.7):

\[
V(r_{ij}) = \frac{1}{4\pi\varepsilon_0} \frac{q_i q_j}{r_{ij}}
\]

**Equation 4.7.**

where \( \varepsilon_0 \) is the permittivity in a vacuum, and \( q_o = -0.8476 \,[\text{e}] \) and \( q_o = 0.4238 \,[\text{e}] \) are the partial charges, respectively. The SPC/E model was proved to accurately describe the self-diffusion coefficient of water in a temperature range around 300 K.\(^{112}\) Lennard-Jones potentials were treated with a twin-range cut-off and 1.5 nm cut-off distance, whereas for electrostatic interactions, a Particle Mesh Ewald (PME) was used with 1.5 nm real-space cut-off, a 0.12 nm reciprocal space gridding, and splines of order 4 with \( 10^{-5} \) tolerance.\(^{111}\)

**USPIO model.** The unit cell of magnetite (Fe\(_3\)O\(_4\)) was here considered (left-hand side of Figure 4.4). The latter unit cell is replicated along all Cartesian
coordinates, such that a fully periodic brick with desired size is constructed (here 2 x 2 x 2 nm$^3$, see middle picture in Figure 4.4). Finally, a spherical particle is obtained by retaining only atoms within a fixed distance from the brick center (radius).

![Figure 4.4. Preparation of a Superparamagnetic Iron Oxide (USPIO) nanoparticle. From left hand side: Magnetite unit cell, periodic brick and final USPIO nanoparticle.](image)

After the above cut, the USPIO surface is treated according to the following steps: 1) Iron atoms Fe$^{2+}$ and Fe$^{3+}$ are removed when they have less than 4 and 6 bonds, respectively; 2) a bonded hydrogen atom is attached to all oxygen atoms with only one bond at the USPIO surface (see Figure 4.5).
Figure 4.5. Surface treatment of USPIOs nanoparticles. From left- to right-hand side: detail of a Fe$^{2+}$-OH group; detail of a Fe$^{3+}$-OH group; whole USPIO particle (2.09 nm diameter) with surface treatment.

For minimizing the electrical dipole of charges at the USPIOs surface, only couple of USPIO nanoparticles were considered for each configuration, with one USPIO in the couple being the mirror image of the other in the same couple (Figure 4.6). In the initial configuration of our simulation, the latter segment is aligned with the pore axis.

Figure 4.6. USPIOs are constructed in pairs. Each particle is the mirror image of the other, with respect to the midpoint. On the right-hand side, a rendered image of a USPIO mirrored pair.
**Force field for USPIOs.** The above USPIO nanoparticles can be described by both harmonic bond stretching and bending angle potentials as follows (Equation 4.8):

\[
V_{Fe}(r_{ij}, \theta_{ijk}) = \frac{1}{2} k_{ij}^b (r_{ij} - r_{ij}^o)^2 + \frac{1}{2} k_{ijk}^\theta (\theta_{ijk} - \theta_{ijk}^o)^2
\]

**Equation 4.8.**

Since the main concern is to study the influence of a number of USPIOs on the water mobility within a silica nanopore, here there was no interest in studying the fast dynamics within the USPIOs and a sufficiently high value for the force constants in Equation 4.6 (rigid particles) was assumed for all bonded interactions with \( k_{ij}^b = 400,000 \) [kJ mol\(^{-1}\) nm\(^{-2}\)] and \( k_{ijk}^\theta = 400 \) [kJ mol\(^{-1}\) rad\(^{-2}\)].

Similarly to silica, non-bonded interactions include Lennard-Jones terms (Equation 4.2, Equation 4.3) and a Coulomb potential (Equation 4.4). Both the adopted parameterization for Lennard-Jones potentials and the partial charges are reported in the following Table 4.3.\(^{113,114}\)
<table>
<thead>
<tr>
<th>Atom</th>
<th>$\sigma_{ii}$ (nm)</th>
<th>$\epsilon_{ii}$ (kJ mol$^{-1}$)</th>
<th>$q_{i}$ (e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$^{2+}$ (1 Fe$^{2+}$-OH group)</td>
<td>0.4300</td>
<td>24.9434</td>
<td>1.21</td>
</tr>
<tr>
<td>Fe$^{2+}$ (2 Fe$^{2+}$-OH groups)</td>
<td>0.4300</td>
<td>24.9434</td>
<td>2.42</td>
</tr>
<tr>
<td>Fe$^{2+}$ (3 Fe$^{2+}$-OH groups)</td>
<td>0.4300</td>
<td>24.9434</td>
<td>3.63</td>
</tr>
<tr>
<td>Fe$^{3+}$ (1 Fe$^{3+}$-OH groups)</td>
<td>0.4300</td>
<td>24.9434</td>
<td>0.73</td>
</tr>
<tr>
<td>Fe$^{3+}$ (2 Fe$^{3+}$-OH groups)</td>
<td>0.4300</td>
<td>24.9434</td>
<td>1.46</td>
</tr>
<tr>
<td>Fe$^{3+}$ (3 Fe$^{3+}$-OH groups)</td>
<td>0.4300</td>
<td>24.9434</td>
<td>2.19</td>
</tr>
<tr>
<td>Fe$^{3+}$ (4 Fe$^{3+}$-OH groups)</td>
<td>0.4300</td>
<td>24.9434</td>
<td>2.92</td>
</tr>
<tr>
<td>Fe$^{3+}$ (5 Fe$^{3+}$-OH groups)</td>
<td>0.4300</td>
<td>24.9434</td>
<td>3.65</td>
</tr>
<tr>
<td>O (Fe$^{2+}$-OH groups)</td>
<td>0.3795</td>
<td>0.5336</td>
<td>-1.61</td>
</tr>
<tr>
<td>O (Fe$^{3+}$-OH groups)</td>
<td>0.3795</td>
<td>0.5336</td>
<td>-1.13</td>
</tr>
<tr>
<td>Fe$^{2+}$ (bulk)</td>
<td>0.4300</td>
<td>24.9434</td>
<td>0</td>
</tr>
<tr>
<td>Fe$^{3+}$ (bulk)</td>
<td>0.4300</td>
<td>24.9434</td>
<td>0</td>
</tr>
<tr>
<td>O (bulk)</td>
<td>0.3795</td>
<td>0.5336</td>
<td>0</td>
</tr>
<tr>
<td>H</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Table 4.3. Parameters for USPIO non-bonded interactions.

In the previous Table 4.3, Fe$^{2+}$ and Fe$^{3+}$ are iron(II) and iron(III) atoms, respectively. Notation ‘Fe$^{m+}$ (n Fe$^{m+}$-OH group)’ indicates an iron(m) atom with n hydroxyl groups at the USPIOs surface, and it is bonded to [(m\times2)-n] bulk oxygen atoms; while ‘O (Fe$^{m+}$-OH group)’ indicates an oxygen atom belonging to a hydroxyl group at the USPIOs surface, and it is bonded to an iron(m) atom. Atoms that do not
belong to any hydroxyl group at the USPIOs surface are referred to as ‘bulk’ atoms. Lennard-Jones potentials were treated with a twin-range cut-off and 1.5 nm cut-off distance, whereas for electrostatic interactions, a Particle Mesh Ewald (PME) was used with 1.5 nm real-space cut-off, a 0.12 nm reciprocal space gridding, and splines of order 4 with $10^{-5}$ tolerance.\textsuperscript{111}

**Water self-diffusion.** The isotropic self-diffusion coefficient $D_w$ of water molecules, is based on the mean square displacement (MSD) and the Einstein relation, namely (Equation 4.9)

$$MSD = \lim_{t \to \infty} \left\langle \left\| \vec{r}_i(t) - \vec{r}_i(0) \right\|^2 \right\rangle_{i \in \text{water}} = 6D_w t$$

**Equation 4.9**

where the position vector refers to the center of mass of the water molecule, 0 is the generic time origin for the reference configuration and $t$ is the generic time instant of actual configuration (6 is a fixed geometrical coefficient due to the derivation of the previous relation from the Langevin equation in the limit of strong friction).

Molecular dynamics simulations have been performed for 1 ns. The steady state is achieved when $D_w$ (evaluated every 200 ps) tend to an asymptotic value. According to the previous criterion, the steady state has been achieved approximately after 600 ps in all the simulations (even before, in the smaller systems). Hence, the isotropic diffusion coefficients of water have been evaluated by
fitting MSD of water from 600 ps to 1000 ps. Different fitting intervals of MSD have been compared for the calculation of the average D and its confidence interval.

Numerical results show that water molecules tend to strongly bind to the surface of the USPIOs, where they undergo a transition into a glassy state with low mobility.\textsuperscript{115,116}

\textbf{4.2.5. \textit{In vitro} guidance of the magnetic nanoconstructs.}

A microfluidic system was used comprising a commercially available parallel plate flow chamber (Glycotech – Rockville, MD, U.S.A.) mounted on a 35 mm cover slip; a syringe pump (Harvard Apparatus, MA) and an epi-fluorescence inverted microscope (Nikon Ti-Eclipse). After proper sonication, the solution of USPIO-loaded nanoconstructs \((10^7 \text{ mL}^{-1})\) was infused in the system with a shear rate of \(25 \text{ s}^{-1}\) (SiMPs). The magnetic guidance was performed by placing a discoidal magnet (D401-N52, K&J Magnetics Inc.) under the cover slip, before the flow was started. Movies were taken during the experiments focusing on different regions of interest up to \(\approx 1000 \mu\text{m}\) away from the magnet. In-flow and drifting velocity have been calculated via offline analysis on the \(x\) and \(y\) displacement of particles in the time interval.

\textbf{4.2.6. Degradation of the SiMPs and USPIO Release.}

The USPIO-loaded SiMPs were exposed to \(1\times\) phosphate buffered saline and left for 3, 6, and 24 h in a shaking incubator at 60 rpm and 37 °C. Samples were
centrifuged at 4000 rpm and the supernatant was assessed for the presence of Fe using ICP-OES.

4.2.7. Relaxometric Analysis.

In vitro relaxation times were measured in a Bruker Minispec (mq 60) benchtop relaxometer operating at 60 MHz and 37 °C. The longitudinal ($T_1$) relaxation times were obtained using inversion recovery pulse sequence. The transverse ($T_2$) relaxation times were measured using Carr-Purcell-Meiboom-Gill (CPMG) sequence. In vitro $T_2$-weighted MR phantom studies were performed in a clinical 3T scanner (Philips Ingenia) using turbo spin echo (TSE) sequence with TR = 2500 ms, TE = 100 ms and a slice thickness of 400 μm. For phantom imaging, a known number of USPIO-loaded SiMPs were embedded in a 1% Agarose matrix.

4.2.8. Cell Line and Tumor Model

B16-F10 cells (from ATCC, Rockville, MD, USA) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U mL$^{-1}$ penicillin and 100 mg/mL streptomycin, and maintained at 37 °C in a 5% CO$_2$ incubator. All the cell culture products were purchased from Invitrogen (Carlsbad, CA, USA). For the tumor model, $10^6$ B16-F10 cells in 200 μL PBS were injected subcutaneously into the flank of 12-weeks old male Nude mice (Nu/Nu) purchased from Charles River (Wilmington, MA, USA). Mice were kept on a 12 h light-dark cycle with food and water ad libitum. All animal experiments in this study
were approved by the Institutional Animal Care & Use Committee (IACUC) of The Methodist Hospital Research Institute.

**4.2.9. *In Vivo* MR Imaging**

10–15 days after tumor implantation, the mice were injected intravenously with either $5 \times 10^8$ 5 nm USPIO-loaded SiMPs or USPIOs alone ($\approx 8 \mu g$) in 150 $\mu L$ of 1× phosphate buffered saline, in the presence and in the absence of an external magnet (D401-N52, K&J Magnetics Inc.), placed on top of the tumor. After 4 h, the magnet was removed and MR imaging were performed at 4 and 24 h post injection. $T_2$-weighted MR images were acquired in a 3T clinical scanner (Philips Ingenia) using spin echo sequence with $TR = 3000$ ms, $TE = 100$ ms, and a slice thickness of 500 $\mu m$. FOV is $80 \times 80$ and reconstructed resolution matrix of $512 \times 512$. MRI images were analyzed using OsiriX imaging software – DICOM viewer (http://www.osirix-viewer.com). All animal experiments performed were in line with the institutional guidelines on the ethical use of animals.

**4.2.10. Histological Analysis**

The mice were sacrificed at 24 h post nanoconstructs injection and their organs were removed and fixed in 10% Formalin for histological study. Transverse sections (4 $\mu m$ in thickness) of paraffin-embedded tumors were stained with the Prussian blue (PB stain), to identify the accumulation of iron oxide; and with hematoxylin and eosin (H&E stain) to identify the presence of the SiMPs in the tissues. Sections were examined with a Nikon Eclipse 80i microscope using a 100×
objective, and digital images were obtained with a CCD camera (Nikon digital sight DS-U3).

### 4.3. Results and Discussion

#### 4.3.1. Assembly of the Magnetic Nanoconstructs

The schematic representations of magnetic nanoconstructs are presented in Figure 4.7a, discoidal mesoporous silicon particles (SiMPs) with a diameter of ≈ 1000 nm and a thickness of ≈ 400 nm and USPIO. USPIOs with a magnetic core diameter of 5 nm and with hydrophilic coatings were examined for the loading and characterization. The size distribution was estimated from the analysis of the TEM images considering more than 100 USPIOs (Figure 4.8).

![Schematic representation of magnetic nanoconstructs](image)

**Figure 4.7.** Magnetic nanoconstructs and USPIOs distribution. a) Ultra small superparamagnetic iron oxide nanoparticles (USPIOs) are loaded within the
porous structure of 1000 nm × 400 nm discoidal silicon particles (SiMPs). Mesoscopic clusters of USPIOs are formed within the porous structure leading to multiscale, hierarchically structured magnetic nanoconstructs. Energy Dispersive X-Ray (EDX)–TEM mapping (b) for the SiMPs. These maps show the distribution of the most abundant element (i.e., silicon (Si) for the SiMPs and the dispersion of 5 nm USPIOs with the corresponding porous matrix (red dots in the third column). Note the formation of multiple, mesoscopic clusters of USPIOs within the matrix (scale bar: 100 nm).

Figure 4.8. TEM images (left) of the USPIOs and size distribution histograms (right).

The USPIOs were loaded within the SiMP pores via capillary action by directly exposing dry SiMPs to a concentrated stock solution of hydrophilic USPIOs. The resulting nanoconstructs were then analyzed using TEM, coupled with energy-dispersive X-ray (EDX-TEM), to confirm USPIO loading and document their spatial distribution within the porous matrix. The EDX mappings of Si and Fe for the SiMPs (Figure 4.7b) showed the fine porous structure of the nanoconstructs, with lateral and internal walls, and a quite uniform distribution of USPIOs (red spots) across the porous matrix. The images in Figure 4.7 eloquently show that the USPIOs tend to
form mesoscopic clusters dispersed throughout the matrix of the resulting magnetic nanoconstruct.

The presence of Fe in the nanoconstructs was also documented by EDX spectral analysis and Magnetic Force Microscopy (MFM). EDX spectra were generated for SIMPs and nanoconstructs upon loading of the USPIOs. These spectra are shown in the Figure 4.9, and present a clear peak for the element iron (Fe), documenting on the effective loading of USPIOs. Huge peaks are observed for the element silicon (Si) for the SiMPs and USPIO/SiMPs as well. The MFM measurements were performed in tapping mode: with the first passage, the probe contacted on the surface returning topographical information (Figure 4.10a, b, d, and e), brighter colours indicate a larger distance from the substrate.; with the second passage, the probe was lifted 35 nm over the sample acquiring for the tip-sample magnetic interactions (Figure 4.10c and f), brighter colours indicate a stronger field and higher USPIO concentration. The contrast between the yellow/brown for c)USPIO/SiMP, and the black magnetic mica substrate is striking, when comparing to free f)SiMP when there is no any observable magnetic difference between the substrate and the particle.
Figure 4.9. EDX spectrums of SiMPs (top) and USPIO-loaded SiMPs (bottom). For the USPIO-loaded particles, the spectrum presents peaks at 0.6, 6 and 7 keV, corresponding to Fe atoms.

Figure 4.10. Images of nanoconstructs captured with MFM mode. Topographical image showing a) USPIO/SiMP and d) SiMPs, tilted with respect to the mica substrate. b) and e) The same image as from a) and d) modified to include the actual dimension of the nanoconstructs. c) and f) MFM image showing the variation of the magnetic field over the nanoconstructs and SiMPs.
The mass of USPIOs loaded per SiMP is given in Figure 4.11a: up to $15.5 \pm 2.5 \times 10^{-9}$ μg of 5 nm USPIOs are loaded per SiMP. Inductively coupled plasma optical emission spectroscopy (ICP-OES) was performed for the element Fe for quantifying the mass of loaded USPIOs.

**Figure 4.11.** USPIO loading and stability of the magnetic nanoconstructs. a) The mass of USPIOs loaded within the mesoporous nanoconstructs is measured via inductively coupled plasma optical emission spectroscopy (ICP-OES). b) The percentage of USPIOs released from a mesoporous SiMP is measured as a function of the incubation time in a buffer solution, agitated at 60 rpm and 37 °C. c–e) Scanning electron micrographs showing the morphology of the SiMPs at three different incubation time points, namely 3, 6, and 24 h. The inset in (e) shows the uneven degradation of the SiMPs that tend to assume a mushroom shape (scale bar: 1.0 μm).
As the USPIO/SiMP nanoconstructs would be later used for \textit{in vivo} studies, it was important to analyze their stability, in that it is well known that porous silicon spontaneously degrades into orthosilicic acid under physiological conditions, thus releasing its payload.\textsuperscript{117,118} A stability test was performed by exposing 5 nm USPIO-loaded SiMPs to a buffer solution, agitated over time at 60 rpm and 37 °C. After 24 h of incubation, only 24.0 ± 2.8% of the originally loaded USPIOs were released upon silicon degradation (Figure 4.11b). SEM images of the magnetic nanoconstructs at different time points during the degradation process, namely 3, 6, and 24 h, are shown in Figure 4.11c–e. Significant changes in the SiMP geometry are visible at 24 h. This is consistent with previous data showing that PEG chains, here decorating the USPIOs, modulate the interaction of the solvent with the silicon walls, thus slowing the degradation process.\textsuperscript{118}

\textbf{4.3.2. Relaxometric Characterization of the Magnetic Nanoconstructs}

It has been recently shown that the geometrical confinement of Gd\textsuperscript{3+}-based MRI contrast agents within mesoporous structures enhances their longitudinal relaxometric response.\textsuperscript{119–121} To study the effect of USPIO confinement within porous matrices, the longitudinal ($T_1$) and transverse ($T_2$) relaxation times of our magnetic nanoconstructs were measured by a bench-top relaxometer operating at 60 MHz (1.41 T) and at 37 °C. The ability of any material to act as a MRI contrast agent (CA) is expressed in terms of its relaxivity $r_i$, defined as the change in relaxation rate of water protons brought about by mM concentration of CA. The relaxivities $r_{1,2}$ of the nanoconstructs were calculated using the classical formula $r_{1,2}$
= (T_{1,2-1} - T_{0-1})/[Fe], T_0 being the diamagnetic contribution, and [Fe] the iron concentration in mM. Upon confinement within a porous matrix, the USPIOs exhibited a significant increase in transversal relaxivity $r_2$ compared to free USPIOs in bulk solution (Figure 4.12a). It increases from 107 ± 24 to 270 ± 73 mm$^{-1}$ s$^{-1}$ for the 5 nm USPIOs (2.5 times) loaded into the SiMPs. On the other hand, no relevant changes are observed for the longitudinal relaxivity $r_1$. A contrast agent is classified as $T_1$-weighted or $T_2$-weighted based on their $r_2/r_1$ ratio, and a $r_2/r_1 > 2$ implies that the agent is more effective as a $T_2$-weighted contrast agent.\textsuperscript{71} A significant increase in the $r_2/r_1$ ratio is observed for USPIOs upon confinement within the porous matrix of the hosting nanoconstructs.

\[
\text{Table 4.12a: Relaxometric characterization of the magnetic nanoconstructs.} \]

<table>
<thead>
<tr>
<th>Samples</th>
<th>$r_2$ (mM$^{-1}$s$^{-1}$)</th>
<th>$r_1$ (mM$^{-1}$s$^{-1}$)</th>
<th>$\Delta r_2$</th>
<th>$r_2/r_1$</th>
<th>$\Delta r_2/r_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 nm</td>
<td>107 ± 24</td>
<td>8 ± 1</td>
<td>2.5 - fold</td>
<td>13 ± 3</td>
<td>2.6 - fold</td>
</tr>
<tr>
<td>5 nm USPIO/SiMPs</td>
<td>270 ± 73</td>
<td>8 ± 2</td>
<td></td>
<td>34 ± 9</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.12. Relaxometric characterization of the magnetic nanoconstructs. a) The transversal ($r_2$) and longitudinal ($r_1$) relaxivities, and the $r_2/r_1$ ratio are listed for the free USPIOs and the corresponding USPIO-loaded nanoconstructs, as derived from a bench-top relaxometric analysis. The change in transversal relaxivity $\Delta r_2$ and $\Delta r_2/r_1$ ratio is also provided, showing a significant enhancement in MRI performance. b) Phantom images for different concentrations of the 5 nm USPIO-loaded SiMPs (magnetic...
nanoconstructs) generated using a 3T Philips MRI clinical scanner. Note that $10^7$ SiMPs are equivalent to $\approx 0.2 \mu g$ of Fe.

The contrast enhancement efficacy of the magnetic nanoconstructs under clinical settings was tested in a 3T MRI scanner (Philips Ingenia). Figure 4.12b shows the phantom images for different numbers of magnetic nanoconstructs embedded in 1% agarose. The phantom images show that the magnetic nanoconstructs are effective under clinical settings, and even a small number of nanoconstructs can generate sufficient contrast.

The observed enhancement in the $r_2$ relaxivity can be ascribed to the hierarchical organization of the USPIOs within the hosting matrix (see TEM and EDX analysis in Figure 4.7 and Figure 4.9). Differently from what is observed for the longitudinal relaxivity enhancement, here, $r_2$ is mostly affected by the formation of USPIO clusters and the so called outer sphere mechanism. In general, the transversal relaxivity $r_2$ increases with the particle magnetization ($\propto M_s^2$) and with the inverse of the diffusion ($\propto D_{w}^{-1}$) of the water molecules surrounding the magnetic core inline image. However, given the well know lower mobility of water molecules within mesoporous structures, and the variety of USPIO clusters generated within the matrices, it is reasonable to speculate that, under such configuration, $D_{w}$ could play a major role (Figure 4.7). To better elucidate the mechanisms regulating the $r_2$ enhancement, a molecular dynamics (MD) model was developed for the USPIO confinement within mesopores. The geometry of the model is depicted in Figure 4.13a, where an individual USPIO is shown first, followed by 1,
4, and 8 couples of USPIOs confined within a periodic slice of a mesopore. The model allows for computing changes in the diffusion of water molecules $D_w$ as a function of the loading conditions (number of USPIOs per pore slice) and surface properties of the USPIOs (polymer chain length and density). Note that the USPIOs form multiple mesoscopic clusters in the nanoconstructs, within which the water molecule mobility is impaired mostly by geometrical constriction (Figure 4.13a). Details about the MD model are provided in the Materials and Methods part. The computed coefficient of diffusion, $D_w$, is presented in Figure 4.13b. The calculations were performed for three strengths of the Lennard-Jones (LJ) potential, in order to explore how the observed behavior depends on the actual properties of the USPIO surface (i.e., different polymer chain length and density). Starting from free bulk water ($D_w = 2.7 \times 10^{-9} \text{ m}^2\text{s}^{-1}$), the coefficient $D_w$ reduces as the number of loaded USPIOs increases, being $\approx 2.1 \times 10^{-9} \text{ m}^2\text{s}^{-1}$ for 1 couple of USPIOs (25% decrease), $\approx 1.4 \times 10^{-9} \text{ m}^2\text{s}^{-1}$ for 4 couples of USPIOs (50% decrease), and $\approx 0.4 \times 10^{-9} \text{ m}^2\text{s}^{-1}$ for 8 couples of USPIOs (85% decrease). Note that $D_w$ is computed by averaging over the pore cross-section, thus even lower water mobilities are expected in the immediate vicinity of the USPIOs, and in the interspaces between the USPIOs and the pore walls. These data show that a 85% decrease in the diffusion of water molecules $D_w$ is likely to occur in USPIOs-loaded nanoconstructs, and this would already justify an increase in $r_2$. Remarkably, even a reduction by an order of magnitude in the LJ potential seems to be accompanied by only a tiny increase in water mobility. Since the strength of the interaction potential is also affected by the adsorption of molecules on the particle surface, the mild effect of the LJ potential on
$D_w$ would suggest that water confinement is governing the relaxivity enhancement. The mechanism proposed above is in agreement with the observations of Gillis and colleagues on the magnetic relaxation properties of USPIO clusters.

![Figure 4.13. Molecular Dynamics simulation for the self-diffusion coefficient of water in a mesopore. a) Molecular Dynamics representation of an individual USPIO, a couple, 4 couples and 8 couples of USPIOs adsorbed on the walls of a mesopore with a hydrated silicon surface. b) Computed diffusivity of the water molecules, averaged over the cross-section of the mesopore, presented as a function of the loading conditions (1, 4, and 8 couples of USPIOs per periodic slice of a mesopore, corresponding to 6%, 24%, and 48% of the maximum geometrical loading) and strength of the Lennard-Jones potential. Error bars indicate 98% confidence intervals based on Student’s t distribution associated with four $D_w$ measurements upon the achievement of steady state conditions. Variable LJ potentials allow one to explore the effects due to the actual surface properties (i.e., different pegylation levels). It is here important to emphasize that transversal relaxivities $r_2$ higher than 300 mm$^{-1}$s$^{-1}$ have been demonstrated with individual, high-performance...
IOs. However, in these cases, the enhancement in relaxivity is mainly due to the improvement of the intrinsic magnetic properties of the nanoparticles rather than the modulation of the local water mobility. Therefore, it is reasonable to speculate that the geometrical confinement of these high-performance IOs within the proposed mesoporous nanoconstruct could further boost their original MRI relaxivities.

4.3.3. Remote Guidance of the Magnetic Nanoconstructs and Cooperative Accumulation

The loading data of Figure 4.11a demonstrate that over $4.0 \times 10^4$ USPIOs are loaded into a single SiMP. This provides a huge, localized concentration of Fe that is equivalent in volume to a spherical bead of several hundreds of nanometers in diameter. In this case, the magnetic guidance of our nanoconstruct under an external static field was tested in a quiescent fluid and under flow, using a parallel plate flow chamber apparatus.$^{105}$

The first characterization of magnetic guidance of 5 nm USPIO-loaded SiMPs was performed under static conditions. 3 µl of solution was deposited on the edge of a rectangular microscope slide. Free USPIOs and USPIOs-loaded SiMPs were tested with a concentration of 0.3 mg/ml and $1.15 \times 10^8$ SiMPs per ml of water, respectively. All samples were sonicated for 2 min and vortexed prior to use. A flat discoidal magnet (25 mm diameter x 1.6 mm thickness, grade N48, from Apex Magnets) was used for the dragging experiments. The magnet generates a field of $\sim 0.2$ T at the center of its circular base. The magnet was placed at three different separation
distances from the drop, namely 1, 3, and 6 mm. Movies were recorded showing the progressive accumulation of the magnetic nanoparticles towards the edge of the magnet. Representative images are shown in the Figure 4.14 and Figure 4.15. The approaching velocity of the nanoconstructs towards the magnet increases as the relative distance between the nanoconstructs and the magnets decreases. (see plots in Figure 4.14). For a magnet placed at 1 mm from the drop edge, the maximum approaching velocity is as high as ≈ 35 µ/s. This reduces to ≈ 10 and 5 µ/s for separation distances of 3 and 6 mm. A similar behaviour was also observed for the free 5 nm USPIO tested under the same experimental conditions. Given the small size of USPIO particles, bright field microscopy does not allow to separate the individual nanoparticles. A film of individual USPIO was observed to build up over time at the edge of the drop next to the magnet (Figure 4.15). The approaching velocity was defined as the rate of growth of this film thickness. This was observed to grow rapidly within the first three minutes. The rate of growth depends on the separation distance between the drop and the magnet as it was as high as ~ 0.6 µm/s for a magnet at 1 mm (see plots in Figure 4.15). Note that the approaching velocity in the case of free USPIOs is significantly lower than for the USPIOs-loaded SiMPs.
Figure 4.14. Magnetic guidance of 5 nm USPIO-loaded SiMPs under static conditions. a) Bright field microscopy images obtained at the different time points showing accumulation of the USPIO-loaded SiMPs at the droplet edge next to the magnet. b) Variation of the approaching velocity for the USPIO-loaded SiMPs as a function of the relative distance between the magnet and the nanoconstructs.
Figure 4.15. Magnetic guidance of 5 nm USPIOs under static conditions. a) Bright field microscopy images obtained at the different time points showing the formation of a thin film of USPIOs at the droplet edge next to the magnet. b) Variation of thickness of the USPIO thin film over time, for three different magnet-to-droplet separation distances.

To further understand the behavior of the USPIO-loaded SiMPs, a second characterization for magnetic guidance was performed using a parallel plate flow chamber. This system reproduces hydrodynamic conditions similar to those experienced in vivo, in the microcirculation. The apparatus comprises a commercially available flow chamber (Glycotech – Rockville, MD, U.S.A.) mounted on a 35 mm cover slip; a syringe pump (Harvard Apparatus, MA); a Nikon Ti-Eclipse epi-fluorescence inverted microscope (Figure 4.16). The flow chamber consists of a poly(methyl methacrylate) (PMMA) deck on top of which there are two slots (inlet and outlet). The first one leads the fluid pumped at constant flow rate from the syringe pump; the second tube carries the waste solution into an empty tube.
Between the flow deck and the coverslip, a rubber gasket is placed that defines the dimension and the geometry of the chamber in which the pumped solution flows, and consequently the flow conditions. For the dragging experiments, the gasket has a thickness $h = 0.254$ mm, a length $l = 20$ mm, and a width $w = 10$ mm. The flow rate has been calculated from the desired shear rate, following the wall stress Equation 4.10.

$$\tau = \mu S = 6\mu \frac{Q}{bh^2}$$

Equation 4.10. $\tau$ represents the wall shear stress; $S$ [s$^{-1}$] is the wall shear rate; $\mu$ [Pa·s] represents the dynamic viscosity of the infused medium; and $Q$ [m$^3$/s] is the flow rate.

Figure 4.16. Parallel flow chamber apparatus. a) The apparatus comprises a syringe pump; a fluorescent inverted microscope and the actual flow chamber; b) flow chamber mounted on the stage of the microscope and connected to the vacuum syringe; c) bottom view of the chamber with the magnet applied directly over the glass slide.
The experiments were performed with $S = 25 \text{ s}^{-1}$, corresponding to $Q \sim 2.69 \mu l/s$. The infused solution has USPIOs-loaded SiMPs in the concentration of $10^7/\text{ml}$. Magnetic guidance experiments were performed placing a discoidal magnet (6.4 mm diameter x 0.8 mm thickness, grade N52, from K&J Magnetics Inc) below the coverslip, halfway of the chamber and on its side. Precisely, the center of the magnet was placed at about 9 mm away from the inlet bore along the flow direction, and about 4.5 mm from the center of the channel on the side. The magnet generates a 0.2 T field at the center of its circular base. The entire system was mounted on the stage of an epifluorescent microscope. The dynamics of the USPIO-loaded SiMPs was monitored over time using the bright field modality. Movies were recorded during the experiments looking at regions of interest located at different distances from the magnet, namely 130, 290 and 1,130 µm. The velocity of the magnetic nanoconstructs was calculated via off-line imaging analysis. The longitudinal and transversal velocities, and the trajectories of the magnetic nanoconstructs for a typical experiment are presented in Figure 4.17. The in-plane nanoconstruct velocity is decomposed in the longitudinal component, $V_x$, aligned with the flow; and the transversal component, $V_y$, orthogonal to the flow and oriented towards the magnet. Figure 4.17b shows the normalized velocity $V_y/(Sh)$ increasing steadily from zero to about 0.002, corresponding to $\approx 10 \mu m \text{ s}^{-1}$, at 50 µm away from the magnet. Similarly, the angle $\theta$ between the flow direction and the particle trajectory varies significantly ranging from 0 to over 30°, as the nanoconstruct approaches the magnet. Only minor changes are observed for $V_x$. The inset of Figure 4.17c shows a dark corona originating from the progressive deposition of nanoconstructs around
the magnet. These pictures were taken on the flow chamber coverslip after magnet removal.

Figure 4.17. Remote guidance of the magnetic nanoconstructs and cooperative accumulation. a) Schematic of the parallel plate flow chamber apparatus used for testing the guidance of the magnetic nanoconstructs under controlled biophysical conditions. The static magnet was placed underneath the chamber, on the side and the nanoconstruct solution was infused via a syringe pump through the inlet bore. b) The variation of the longitudinal and transversal components of the nanoconstruct velocity, $V_x$ and $V_y$, normalized with the chamber height $h$ and wall shear rate $S$; and of the angle $\theta$ between the flow direction and the particle trajectory with the separation distance from the magnet. c) Image of a dark corona originating from the progressive accumulation of nanoconstructs around the magnet.

In order to better characterize the mutual interaction of the nanoconstructs under static magnetic fields, experiments under flow were also performed in the presence of small clusters of nanoconstructs pre-formed on the bottom of the flow
chamber (green dotted circles in Figure 4.18 at \( t = 0 \) min). A few drops of a solution containing the nanoconstructs were deposited on the coverslip and left to dry in air, while a static magnet was placed underneath the coverslip. After complete drying, the coverslip with the pre-formed clusters was assembled with the rest of the parallel plate flow chamber apparatus and the actual experiment was performed using a fresh solution of the nanoconstructs. USPIO-loaded SiMPs were then injected in the parallel plate flow chamber and the size of the four representative clusters was monitored over time (Figure 4.19). These experiments were performed in the presence of an external magnetic field and, as a control, without any field. Figure 4.18 show microscopy images of the region of interest at the bottom of the chamber starting at the initial time (\( t = 0 \) min) until the end of the experiment. The relative variation of the area associated with four representative clusters (\( \Delta A\% \)) is plotted as a function of time in Figure 4.19. The area was computed using ImageJ and defined as Equation 4.11.

\[
\Delta A \% = \frac{A(t) - A_0}{A_0} \times 100
\]

**Equation 4.11.** \( A(t) \) is the cluster area at time \( t \) and \( A_0 \) is the initial cluster area at time \( t = 0 \) sec.

The areas of these clusters continuously grow over time, demonstrating the progressive accumulation of individual nanoconstructs around the pre-formed clusters. No variation in cluster size and numbers was observed in the control experiments, when a magnet was not used (Figure 4.20).
Figure 4.18. Accumulation of magnetic nanoconstructs under flow. Bright field microscopy images of USPIO-loaded SiMPs depositing over time on the bottom of a parallel plate flow chamber (S = 15 sec⁻¹), and reconstruction of the clusters formed by the SiMP nanoconstructs using ImageJ (http://rsb.info.nih.gov/ij/).

Figure 4.19. Relative area increase of the nanoconstruct clusters under flow. Quantification of the relative area increase for the four USPIO-loaded SiMP clusters. The area of the clusters is extrapolated from the experimental data of Figure 4.18.
Figure 4.20. Accumulation of magnetic nanoconstructs in the absence of external magnetic field. No difference in cluster area and number of newly formed clusters is observed for USPIO-loaded SiMPs in the absence of a static magnetic field.

Dragging experiments for the USPIO-loaded SiMPs were also conducted under static conditions in a multi-well plate. In this case, the SiMP nanoconstructs, loaded with 5 nm USPIOs, were suspended in 100 µl of DI water and deposited in a multi-well plate (8 wells per row). Then, the dragging of the SiMP nanoconstructs towards the magnet (at the left) was monitored over time (Figure 4.21). The diameter of the wells is about 1 cm, and the magnet was placed at 2 cm away from the multi-well plate. In the upper row of the plate, all the wells were filled with the same nanoconstruct solution. In the lower row, only the last well on the right was filled with the nanoconstruct solution. At time \( t = 0 \) hr, the SiMP nanoconstructs are uniformly dispersed within all the wells with no indication of preferential accumulation. After 8 h exposure, it is observed: i) on the upper row only, the preferential accumulation of the SiMP nanoconstructs on the left side of the well (i.e. towards the magnet) (darker regions); ii) on the lower row, no preferential accumulation of the SiMP nanoconstructs. This would imply that the magnetic field does not affect the nanoconstructs located in the far right well (at 9.2 cm away from
the magnet), unless other nanoconstructs are dispersed in between as for the upper row; iii) on the upper row only, the intensity of the darker region appears quite similar for most of the wells.

**Figure 4.21. Accumulation of magnetic nanoconstructs under static conditions.** At time $t = 0$ hr (top), the SiMP nanoconstructs are uniformly dispersed within the wells. After 8h exposure to a magnet (left), only the SiMPs on the upper row accumulate on the left side of the well attracted by the external field.

The magnetic nanoconstructs, exposed to remote magnetic fields, tend to behave locally as small, non-permanent magnetic dipoles. This would enhance locally the magnetic field and its gradient favoring particle-particle mutual interaction and attraction. This cooperative mechanism is also expected to operate
in vivo, favoring the progressive accumulation of nanoconstructs, particularly in the smaller blood vessels.

4.3.4. MR Imaging in Orthotopic Mouse Models of Melanoma

The MRI and cooperative accumulation properties are demonstrated in vivo for the SiMPs. The in vivo performance of SiMPs has been extensively documented in different animal models for optical imaging and therapy.\textsuperscript{102-104} In particular, it has been shown that discoidal SiMPs of 1000 nm × 400 nm can reach tumor accumulation levels of up to 5\% of the injected dose per gram tissue.\textsuperscript{103} This results from the specific selection of the particle size and shape (i.e., particle geometry) that favors vascular deposition by increasing the interfacial adhesion interactions and reducing the dislodging hydrodynamic forces.

B16-F10 cells were grown in the flank of a mouse for 10 – 15 days before the injection of 5.0 × 10\textsuperscript{8} nanoconstructs in 150 μL of phosphate buffered saline via tail vein. This dose corresponds to ≈ 8 μg of USPIOs per mouse (i.e., ≈ 0.5 mg of Fe kg\textsuperscript{-1}). Three different groups were considered: i) free 5 nm USPIOs with an external magnet (n = 3 mice); ii) nanoconstructs without an external magnet (n = 3 mice); and iii) nanoconstructs with an external magnet (n = 6 mice). The magnet was placed on the tumor side for 4 h post injection and the comparison among the three groups was performed fixing the total amount of injected iron. The mice were imaged pre-injection as a control, at 4 h, after the removal of the magnet, and at 24 h post injection, just before sacrifice using a 3T MRI clinical scanner. Figure 4.22 shows MR images and intensity ratios at the three different time points, and for the
three experimental groups. The tumor is visible on the right flank of the animal. The top row is for the free USPIOs (Figure 4.22a–d), the middle row is for the nanoconstructs without exposure to a magnet (Figure 4.22e–h), and the bottom row is for the nanoconstructs exposed to a magnet (Figure 4.22i–l). In this last case, a significant enhancement in MRI contrast is evident with the appearance of large, dark spots within the tumor mass (Figure 4.22j, k), which are not observed in the case of free USPIOs. The level of nanoconstruct accumulation has been also quantified by considering the intensity ratios over two different regions of interests (ROIs), namely the entire tumor mass (whole tumor mass, in Figure 4.22) and the dark spots within the tumor mass (CA Accumulated Area, in Figure 4.22). The intensity ratios (Figure 4.22d, h, l) are calculated as the difference between the intensity of the ROI and reference water divided by the intensity of the reference water. For the nanoconstructs dragged by a magnet (Figure 4.22l), a significant drop in intensity ratio, up to 60%, was observed within the first 4 h for both ROIs. In the absence of an external magnet, no significant changes in contrast was appreciated by looking directly at the MRI slides, but a slight decrease in intensity ratio (≈20%) can be computed after 24 h (Figure 4.22h). For the free USPIOs in the presence of an external magnet, no statistically significant difference in intensity ratio is observed (Figure 4.22d). Note that the intensity ratios were quantified over multiple planes.
Figure 4.22. MR imaging of the magnetic nanoconstructs accumulating in melanoma bearing mice. a–c) MR images of a melanoma tumor growing in the right flank of a mouse before, 4 h, and 24 h post injection of free 5 nm USPIOs, in the presence of a static magnet applied over the tumor. d) Intensity ratios at tumor region of interest (ROIs) estimated at 0, 4 and 24 h post injection. e,g) MR images of a melanoma tumor growing in the right flank of a mouse before, 4 h, and 24 h post injection of magnetic nanoconstructs, in the absence of a static magnet applied over the tumor. h) Intensity ratios for two ROIs estimated at 0, 4 and 24 h post injection. i–k) MR images of a melanoma tumor growing in the right flank of a mouse before, 4 h, and 24 h post injection of magnetic nanoconstructs, in the presence of a static magnet applied over the tumor. l) Intensity ratios for two ROIs estimated at 0, 4, and 24 h post injection. Note that the intensity ratios have been calculated by averaging the MRI signal over multiple z-planes. A 3T Philips MRI clinical scanner was used. B16-F10 cells were grown in the flank of a mouse for 10–15 days prior injection of 5.0 × 10^8 nanoconstructs via tail vein. This dose corresponds to ≈ 8 μg of SPIOs per mouse (i.e., ≈ 0.5 mg of Fe kg⁻¹).
The absence of any significant contrast for the free USPIOs has to be ascribed to the low dose of iron injected (≈0.5 mg of Fe kg\(^{-1}\)). Indeed, this is at least 20 times lower than the doses commonly used in similar animal experiments and in clinical practice (10 mg of Fe kg\(^{-1}\))\(^{87}\). However, for the same doses, the magnetic nanoconstructs can induce a significant change in contrast, especially upon exposure to a static magnetic field. The intensity ratio clearly demonstrates that the contrast enhancement occurs mostly within the first 4 h, during which the magnet is applied next to the tumor. It should also be emphasized that, given the size of the nanoconstructs, these are not expected to distribute throughout the tumor tissue to provide a uniform darkening of the tumor mass, but would rather accumulate within the tumor microvasculature as explained above. Thus, the significant darkening observed in Figure 4.22j, k, and documented over multiple z-planes within the tumor mass (Figure 4.23), should be related to the progressive accumulation of magnetic nanoconstructs, operating locally as vascular magnetic dipoles and attracting over time other nanoconstructs passing nearby. Dipole–dipole magnetic interactions arising among the nanoconstructs exposed to external static fields would contribute to their progressive deposition within the diseased vasculature and represent a novel, \textit{in vivo} effective inter-particle communication mechanism. Additional MR images are presented in Figure 4.24 for other mice used in this study.
Figure 4.23. MR images acquired over multiple planes pre-injection, 4 and 24h post injection of 5 nm USPIO-loaded SiMPs in the presence of an external magnet.
Figure 4.24. MR images of 5 nm USPIO-loaded SiMPs acquired at 4h post injection over multiple planes and in the presence of an external magnet. Images for three different mice are presented.
Histological sections of the tumor, liver, and spleen are shown in the Figure 4.25 and Figure 4.26, for two different stains, namely classical hematoxylin and eosin (H&E) stain and Prussian Blue (PB) stain. SiMPs appear as black dots in the H&E slides, with a diameter of ≈ 1 μm. In the PB stained slides, the SIMPs appear darker than the surrounding tissue and are surrounded by bluish glows deriving from the Prussian blue staining of the iron. This demonstrates that the USPIOs are still associated with SiMPs. Even for the histological sections, it should be emphasized that, given the size of the nanoconstructs and the association of the USPIOs with the SiMPs, the PB staining is not expected to appear as uniform bluish layer coating the tissue slide but rather as discrete blue spots comparable in size with the SiMPs.
Figure 4.25. Representative histological sections for the tumor, liver and spleen tissues with hematoxylin and eosin (H&E) stain, showing the presence of SiMPs in the tissue. Arrows point at SiMPs in the tissue. Note that the size of the SiMPs is comparable with ≈ 1 µm. Images are taken with a histological microscope using a 100X objective.
Figure 4.26. Representative histological sections for the tumor, liver and spleen tissues with Prussian Blue (PB) stain to identify the presence of USPIOs (blue glow). Arrows point at SiMPs in the tissue. Note that the size of the SiMPs is comparable with $\approx 1 \, \mu m$. Images are taken with a histological microscope using a 100X objective.

4.4. Conclusions

In summary, a novel class of magnetic nanoconstructs has been developed that fully capitalize on the multifunctional properties of USPIOs. The proposed
strategy is of broad applicability and decouples the IO functionality from their geometrical properties allowing the utilization of small, rapidly biodegradable 5 nm USPIOs for diverse functions. Cooperative accumulation at the target site, effective magnetic guidance, traditionally limited to large bulky particles, and MRI contrast enhancement, 2.5-fold higher than conventional clinical systems, have been demonstrated by dispersing mesoscopic clusters of 5 nm USPIOs within larger porous matrices made from silicon. The resulting magnetic nanoconstructs are capable of providing significant contrast already at iron doses 1 to 2 orders of magnitude smaller than current practice. This work also continues to prove that enhancement in relaxivity associated with the geometrical confinement of MRI contrast agents in mesoporous structures is an universal phenomenon, independent of the type of agents. This approach could be used to further boost the already high relaxivities of iron nanocubes and doped iron oxide nanoparticles. The cooperative tumor accumulation of these nanoconstructs could be also used for triggering the release of large amounts of therapeutic cargos directly at the site of interest, and enabling thermal ablation therapies via systemic administration of iron oxide nanoparticles.
References


(54) Richard, C.; Doan, B.-T.; Beloeil, J.-C.; Bessodes, M.; Tóth, É.; Scherman, D. Noncovalent Functionalization of Carbon Nanotubes with Amphiphilic Gd $^{3+}$


