1. Introduction

Carbon nanotubes (CNTs) have great promise for numerous technological as well as biomedical applications, which arise from their unique architecture and their outstanding mechanical and electronic properties. However, their toxicity and fate in the organism is still a subject of debate. The lack of consensus on a range of toxicology and biodistribution issues regarding CNTs is due, on one part, to the wide heterogeneity of CNTs used in terms of length, diameter, number of walls, purity (e.g., the presence of catalysts), morphology, functionalization, and aggregation state. An additional confounding factor is the limited range of methods available to study CNTs, and more generally carbon-based materials, in biological environments. As pristine CNTs are water insoluble, a variety of chemical strategies have been explored to functionalize the nanotube surface and render them dispersible in aqueous media.
media. The type of surface modification, for example, physical coating as opposed to covalent chemical functionalization, has been shown to play a critical role in the interactions of CNTs with cells, in their behavior in the blood compartment and in their subsequent fate in vivo. In particular, oxidation and shortening of multi-walled carbon nanotubes (MWCNTs) by acid treatment followed by chemical functionalization of the hydrophobic carbon structure with hydrophilic amino groups has led to a new generation of functionalized MWCNTs with high dispersibility, low toxicity, high cellular uptake, and favorable pharmacokinetics for medical use. Although functionalized MWCNTs have very promising applications, as they can serve as multifunctional platforms for tumor targeting and drug delivery, therapeutic hyperthermia, transfection of siRNA, and tissue engineering, imaging of MWCNTs in vivo remains a major challenge.

Under controlled conditions imaging of CNTs in biological environments can be achieved using the intrinsic properties of the nanotubes, such as light absorption and scattering, acoustic or photoacoustic properties, Raman signature or electron microscopy. However, with current diagnostic imaging modalities, exogenous labels (mainly fluorescent probes and radionuclides) are usually required for efficient non-invasive tracking of CNTs in living animals.

Among non-invasive imaging methods, magnetic resonance imaging (MRI) appears particularly advantageous because of its excellent spatial resolution and soft-tissue contrast providing simultaneously anatomic, functional and molecular information. The main advantage of MRI over other modalities, such as fluorescence or nuclear imaging, is its ability to penetrate deep into tissue without employing ionizing radiation. In addition to the enormous and ongoing growth in its application in routine clinical practice, MRI has also proven to be a method of choice for molecular imaging, providing cell tracking and direct information on molecular events through the use of smart contrast agents. To date there have been few reports on the use of MRI for visualization of CNTs due to the poor MR contrast generated by CNTs. Recently the magnetic properties of metal impurities present in raw commercially available single-walled CNTs (mainly iron and nickel catalysts) were exploited to investigate CNT distribution with hyperpolarized 3He and conventional 1H MRI. However, the intrinsic T2 and T1 contrast that are exploited in this approach are highly dependent on the quality of purification, on the type of CNTs, and indeed on the aggregation propensity in physiological media all factors which are not easily controllable. Alternatively, CNTs may be coupled to exogenous contrast agents. These agents may allow both, the T2 contrast and signal reduction, as for example is the case of CNT conjugates with iron oxide superparamagnetic nanoparticles or provide T1-based contrast and signal enhancement when the tubes are conjugated to paramagnetic gadolinium. The CNT-contrast agent complexes might be either made by loading the tube cavity or by functionalization of their carbon backbone. For the goal of monitoring the behavior of CNTs in vivo, it is important that any exogenous agent does not influence either the CNT biodistribution, or their ability to transport and deliver a therapeutic agent to an identified target. In this regards both external coupling to or internal loading of CNTs with iron oxide nanoparticles may partially modify their fate in vivo, as the organism must degrade inorganic nanoparticles in addition to carbon materials, which are both slow processes. Alternative strategies such as nanoscale confinement of large quantities of Gd within ultra-short SWCNTs through sidewall defects or at the opened ends (so called gado-nanotubes), synthesis of SWCNT-Gd oxide complexes by use of GdOx catalytic nanoparticles or covalent adsorption of Gd chelates onto MWCNT surface have been shown to produce agents with very high r1 relaxivity (relaxation enhancement per millimolar concentration of agent). However, non-covalent approaches have the disadvantages of saturating the loading capacity of CNTs or strongly reducing the possibility of further functionalization. In addition, most of these Gd-CNT complexes have unknown behavior in vivo, especially after intravenous injection due their poor dispersibility. Only one recent study described intravenous administration of a low dose of SWCNTs, synthesized using gadolinium nanoparticles as catalysts, however an in vivo MRI follow-up was not reported and the fate of gadolinium nanoparticles is not known.

For this purpose, oxidized MWCNTs were covalently functionalized with the chelating molecule diethylenetriaminepentaacetic dianhydride (DTPA), followed by chelation to Gd. As our approach is based on covalent bonding between CNTs and DTPA, which has a high association constant for Gd, the chelation of Gd by DTPA/CNTs is strong, resulting in stable Gd-CNT conjugates and thus reducing the risk of Gd release. The magnetic properties and magnetic resonance relaxivities of the Gd-CNTs in comparison to oxidized CNTs showed the paramagnetic contribution of Gd-chelates which led to positive MR contrast. Remarkably these nanotubes were internalized by cells without toxicity and the positive contrast effect was preserved following cell internalization. Finally, the potential for in vivo monitoring of nanotube fate following intravenous administration of Gd-CNTs in mice was assessed demonstrating that MRI can be used as suitable alternative imaging technique to track CNTs in vivo.

2. Results and Discussion

2.1. CNT Functionalization

MWNTs with a diameter of 20–30 nm functionalized with a DTPA derivative were prepared using a four step procedure (Figure 1). Pristine purified MWNTs were first oxidized in acidic conditions to shorten the tubes to an average length of ~400 nm and to introduce COOH groups (Supporting Information Figure S1). The carboxylic acids were activated using oxalyl chloride. The corresponding acyl chloride groups were then coupled to the DTPA derivative 2 bearing a free amine function (Supporting Information Figure S2). The tert-butyl ester moieties were subsequently hydrolyzed
using trifluoroacetic acid (TFA). Finally, chelation of Gd$^{3+}$ was achieved by dispersing the DTPA/CNTs 4 in an aqueous solution of gadolinium(III) perchlorate, followed by dialysis against water to remove free gadolinium. The usual approach to conjugation of molecules with DTPA involves the conversion of one of the four carboxylic acids to an amide functionality by the use of DTPA dianhydride. In addition to the disadvantage of being non-selective, due to the concomitant formation of diamide derivatives, this method results in conjugates with lower chelating capacity for gadolinium.\cite{29} In our case the DTPA derivative 2 has the advantage of bearing five free COOH groups available for chelation of gadolinium.

The Gd-CNTs were characterized by thermogravimetric analysis (TGA), inductively coupled plasma atomic emission spectrometry (ICP-AES), high resolution transmission electron microscopy (HR-TEM) and scanning transmission electron microscopy (STEM) coupled to energy-dispersive X-ray spectroscopy (EDX). TGA in N$_2$ of pristine MWCNTs, oxidized MWCNTs (ox-CNTs) 1 and DTPA/CNTs 4 showed an increasing weight loss, confirming the efficiency of the functionalization (Figure 2A). The weight loss difference at 500 °C between ox-CNTs 1 and DTPA/CNTs 4 provides an assessment of the level of functionalization; the DTPA loading was estimated 540 µmol per gram of CNTs. The level of functionalization was also determined spectrophotometrically using xylenol orange.\cite{30} For this purpose, after chelation of DTPA/CNTs 4 with gadolinium(III) perchlorate and before filtration and dialysis, the free Gd (i.e., not chelated to DTPA/CNTs) was titrated. The dye xylenol orange is used as complexometric indicator of gadolinium as it shows a color change in the presence of free Gd. This colorimetric test allowed us to determine that the amount of bound gadolinium was 485 µmol per gram of CNTs 5, which is consistent with the value obtained from TGA. The xylenol orange test was also performed to ensure that no free Gd$^{3+}$ was present in the Gd-CNT samples. Control reactions were performed using the precursor CNTs (i.e., ox-CNTs 1). In this case, we did not observe a significant complexation of Gd$^{3+}$ ions by the carboxylic acid functions or the nanotube sidewall. Finally, ICP/AES of Gd-CNTs 5 gave a more precise value of the functionalization degree; 460 ± 4 µmol of gadolinium per gram of nanotubes. Overall, characterization of the CNT conjugates by ICP/AES, TGA, and xylenol orange test was also performed to ensure that no free Gd$^{3+}$ was present in the Gd-CNT samples. Control reactions were performed using the precursor CNTs (i.e., ox-CNTs 1). In this case, we did not observe a significant complexation of Gd$^{3+}$ ions by the carboxylic acid functions or the nanotube sidewall.

2.2. Magnetic Characterizations

Magnetic characterization confirmed the paramagnetic properties of Gd-CNTs 5 in comparison with the ox-CNT precursor 1. The field-dependent magnetization curve at 5 K (Figure 3A) was fit using a Brillouin function characteristic of paramagnetic materials, with a J angular momentum of 3.2. This gives an effective magnetic moment of 6.9 µB, close to the theoretical magnetic moment of Gd$^{3+}$ ($\mu_{eff} = 7.94$ µB, J = 7/2). However, field-cooled (FC) and zero-field-cooled (ZFC) magnetization measurements (using magnetic fields of 100 and 1000 Oe for the former) revealed an additional ferromagnetic component (Figure 3B) which is added to the paramagnetic contribution arising from gadolinium. The FC and ZFC magnetization curves were not strictly superimposable as expected for purely paramagnetic behavior (inset in Figure 3B). Interestingly, the
low temperature ferromagnetic component (with a blocking temperature of about 65 K) was also displayed by the gadolinium-free control nanotubes (ox-CNTs) (Figure 3C). This suggests that the ferromagnetic contribution arises from residual catalyst impurities (620 ppm of iron and 1283 ppm of nickel), which are probably entrapped into the inner empty cavity of CNTs and were not completely eliminated by the acid treatment, for both ox-CNTs and Gd-CNTs. The chelation of gadolinium into DTPA/CNTs gives rise to the stronger paramagnetic contribution, demonstrated by the linear dependence of magnetization with inverse temperature.

MRI contrast agents (CAs) act as enhancers of longitudinal and transverse local $^1$H relaxation rates $R_1 = 1/T_1$ and $R_2 = 1/T_2$. The efficacy of a gadolinium-based CA is measured by its relaxivities $r_i$ and $r_2$ defined as $r_i = (R_i - R_{i0})/[\text{Gd}]$, where $i = 1, 2$ and $R_{i0}$ is the endogenous relaxation rate (Figure 3D). Gd-DTPA (Magnevist, Bayer Schering Pharma) is one of the most common clinically approved CAs used in the majority of clinical procedures. It has $r_1$ value of 4.1 s$^{-1}$ mM$^{-1}$ and $r_2$ of 4.6 s$^{-1}$ mM$^{-1}$ at an imaging field of 1.5 T (or 60 MHz $^1$H Larmor frequency). A relaxivity ratio $r_2/r_1$ of close to 1 ensures that the CA acts as a good local signal enhancer (a positive CA), particularly in $T_1$-weighted sequences. Here, the MR properties of Gd-CNTs are compared to those of a Gd-DTPA chelate we synthesized (Supporting Information Figure S3). The longitudinal relaxivities, $r_1$, were measured at 25 °C over a large range of $^1$H Larmor frequencies (from 0.01 to 200 MHz). Interestingly, the resulting nuclear magnetic relaxation dispersion (NMRD) profile of the Gd-CNT suspension is distinctly different from that of non-conjugated Gd-DTPA, with $r_1$ values enhanced by a factor of two at low frequency (Figure 3E). The decrease of $r_1$ at clinical fields above 10 MHz is less marked for the Gd-CNTs in comparison to Gd-DTPA. However, the result is a three-fold increase in $r_1$ at 200 MHz (4.7 T) due to conjugation to CNTs. This effect could be due to an enhancement of the inner-sphere relaxivity mechanism by slowing down the tumbling of Gd-chelates which exhibit fast exchange of the coordinated water molecules. This characteristic effect has been achieved before by binding Gd ions to objects of increasing size from macromolecules to proteins or nanoparticles.[33] In our case, the nearly one-dimensional structure of functionalized CNTs together with their high dispersibility, highly hydrated surface and high permeability to water may favor water exchange, while increasing the correlation time for tumbling of the whole conjugate. Importantly the huge increase of low field relaxivity demonstrated by non-covalent confinement of Gd-complexes or gadolinium oxide into nanosystems permeable to water, such as zeolites, apoferritin, silicon microparticles and single-walled carbon nanotubes was not observed.[23a,34] This is consistent with an external covalent coupling of water-accessible Gd-chelates at the surface of the oxidized nanotubes. It should also be noted that we did not observe variations of relaxivity with
CNT concentration, as previously reported. All relaxation rates showed linear variation with CNT and Gd concentration, with identical $r_1$ values obtained irrespective of whether the Gd-CNTs were dispersed in water or immobilized in agarose gel (Figure 3 D). The former observation confirms both the remarkably high dispersibility of the functionalized CNTs and their colloidal stability in water suspension over the concentration range studied. The latter suggests that further increases in the overall tumbling correlation time (in agarose) have no effect, and so this is no longer the $r_1$-limiting process. Once bound to the CNT it seems that the timescale for local dynamics about the chelate determines the relaxivity.

Transverse relaxivities $r_2$ were measured in the frequency range between 20 MHz (0.47 T) and 200 MHz (4.7 T). The values of $r_2$ of Gd-CNTs and Gd-DTPA decreased from 13.0 s$^{-1}$ mM$^{-1}$ and 5.40 at 20 MHz to 5.22 s$^{-1}$ mM$^{-1}$ and 4.07 s$^{-1}$ mM$^{-1}$ at 200 MHz, respectively. This change corresponds to an evolution of the $r_2/r_1$ ratio from 1.8 to 0.7 for Gd-CNTs and from 1.42 to 1.40 for Gd-DTPA when the imaging field is increased from 0.47 to 4.7 T. Hence, in terms of $r_2/r_1$ ratio, the efficacy as a positive CA is enhanced for Gd-CNTs on increasing the field, in comparison to Gd-DTPA. Therefore, for the in vitro and in vivo evaluation of Gd-CNTs, an imaging field of 4.7 T was used.

2.3. Cellular Uptake and MRI Cell Imaging

As intrinsic MR contrast highly depends on tissue structure and water dynamics, the ability of a CA to modify the $^1$H signal is strongly affected by the local environment and by CA organization in this environment. During their odyssey through the organism, CNTs are likely internalized by cells, and most probably by cells of the reticulo-endothelial system (resident macrophages or circulating phagocytic cells). However, the magnetic properties of MRI contrast agents are deeply affected by cellular internalization and intracellular compartmentalization. For instance it is well-known that there is a drastic reduction of the longitudinal relaxivity of iron oxide nanoparticles following cellular uptake. Similar effects were observed for gadolinium oxide nanoparticles depending on the internalized dose and for a macromolecular Gd(III)-based probe depending on its intracellular localization. This so-called intracellular “quenching” effect tends to enhance $r_2/r_1$ ratio, in such manner
that the generation of positive contrast by labeled cells becomes challenging. Regarding CNTs, it is not clear how $r_1$ could be modified by cell confinement and how intrinsic properties of precursor ox-CNTs could affect transverse and longitudinal relaxation in that environment. It was reported that, despite a very high $r_1$ value in suspension, gadolinium-loaded SWCNTs failed to induce positive contrast when internalized by murine macrophages and were subsequently detected with a negative signal on $T_2$ or $T_2^*$-weighted sequence. Likewise, CNTs that were free of any gadolinium label were found to induce $^1$H signal suppression in mouse liver. These confusing results call for a thorough examination of MR properties depending on cellular uptake and on the intrinsic magnetic properties of CNTs.

In this work, RAW 264.7 mouse macrophages were used as a cell model to study Gd-CNT uptake in vitro. The cells were incubated for 24 h with Gd-CNTs in the concentration range of 0–100 µg/mL. Cell morphology and adherence on culture flasks was not affected by CNT labeling as shown by optical microscopy (Figure 4A). Cellular metabolic activity, assessed by the Alamar blue test, revealed a slight decrease in comparison to non-labelled cells at the highest concentration tested (100 µg/mL) (see Supporting Information Figure S4). TEM confirmed the intracellular uptake of CNTs; indeed elongated hollow structures were easily identifiable within the intracellular organelles (Figure 4B). Most nanotubes were assembled as large bundles within vesicular membranes, only a small fraction could be observed isolated within the cytoplasm. This suggests an active process of cellular internalization, involving membrane trafficking and dynamic compartmentalization within lysosomal storage organelles. The dose dependent CNT uptake by macrophages was assessed by imaging flow cytometry (ImageStream). This technique combines the statistical power of high throughput flow cytometry with imaging of each analyzed cell. As illustrated in Figure 5A, Gd-CNT-labeled cells exhibit dark spots on bright field images that were not observed in non-labeled cells. These dark spots colocalized with high intensity spots on the dark field image, corresponding to areas of CNT accumulation that strongly absorb and scatter the light. The biparametric dot plot representing the mean intensity on dark field versus the mean pixel intensity on bright field (Figure 5B) clearly shows that both parameters increase (in absolute value) with increasing concentration of CNTs in the incubation medium. This indicates a dose dependent CNT uptake for the entire cell population, which tends to saturate at the highest concentration. Similar results were obtained for both Gd-CNTs and ox-CNTs.

Subsequently the MR contrast properties of cells labeled with increasing concentration of Gd-CNTs or ox-CNTs and dispersed in agarose gels mimicking the cell density of tissue ($7 \times 10^6$ cells in 70 µL of gel) were examined. MR images were acquired using a spin echo sequence, while varying the echo time $T_E$ or repetition time $T_R$ in order to determine $T_1$ and $T_2$ values and the optimal conditions for $T_1$- or $T_2$-weighting. Representative $T_1$-weighted images in Figure 6A show a dose-dependent signal increase in the macrophages labeled with Gd-CNTs in comparison to the non-labeled cells or to the cells labeled with ox-CNTs. The signal increase was nearly twofold for the highest Gd-CNT concentration with respect to non-labeled cells. Conversely, in $T_2$-weighted images, the macrophages labeled with Gd-CNTs display decreased MR signal intensity with increasing CNT concentration (Figure 6A). Hence, the Gd-CNTs act as a bimodal contrast agent for cell imaging, generating positive contrast under $T_1$-weighted and negative contrast under $T_2$-weighted imaging conditions. By comparison, no signal change was...
observed for macrophages labeled with ox-CNTs. The quantitative determination of relaxation rates (1/T<sub>i</sub> expressed in s<sup>-1</sup>, i = 1,2) reveals a linear increase of 1/T<sub>1</sub> with Gd-CNT concentration in macrophages, whereas no variation was observed after labeling with control ox-CNTs (Figure 6B). This shows that Gd-DTPA chelates covalently coupled to CNTs can still be effective as T<sub>1</sub>-contrast agents when internalized in cells. Regarding the transverse relaxation, the analysis is more complex. In comparison to cell-free agarose, the high density of cells induces a 3-fold increase in transverse relaxation time 1/T<sub>2</sub> (Figure 6B). This observation is consistent with relaxation dynamics in tissue, which is accelerated in comparison to water. More surprisingly, 1/T<sub>2</sub> also increased with the concentration of CNTs in cells, regardless of whether they were labeled with Gd-CNTs or ox-CNTs. The T<sub>2</sub> effect appears as an intrinsic characteristic of CNTs, not of the complexed gadolinium probe. In summary covalent grafting of Gd-DTPA induces an efficient intracellular T<sub>1</sub> reduction that depends on CNT cellular uptake. Despite the associated T<sub>2</sub> reduction in labeled cells arising from the nanotubes, positive cell contrast due to the paramagnetic label can be observed when appropriate T<sub>1</sub>-weighting is applied.

2.4. In Vivo Imaging

The next step was to evaluate the use of Gd-CNTs in vivo to monitor the organ biodistribution of functionalized nanotubes. Gd-CNTs were injected intravenously to mice at two different doses (50 µg and 250 µg corresponding to 2.5 and 12.5 mg/kg, respectively) and monitored over 5 hours following injection. A T<sub>1</sub>-weighted sequence (fast spin echo) was applied since it offered the best contrast-to-noise ratio after Gd-CNT administration. Following the injection of the low dose of Gd-CNTs, significant signal changes in liver, spleen, kidneys and bladder were not observed. However a five-fold increase in Gd-CNT dose (250 µg corresponding to a gadolinium dose of 5.75 µmol/kg body weight) yielded a clear signal enhancement in liver, spleen and to a much larger extent in the bladder, where signal enhancement in excess of +250% was obtained (Figure 7). While the contrast enhancement decreased after 2 h in the bladder (Figure 7C), it was slightly increased over time in liver reaching a value of +27% after 320 min (Figure 7AB). In spleen the signal reached a maximum after two hours (+16%) and then decreased to close to its initial value (+3%). Histological specimens of hepatic and splenic tissues confirmed the presence of nanotubes with a spotty homogenous distribution in liver and in the red pulp periphery of the spleen (Figure 8 and Supporting Information Figures S5,S6). Unambiguous identification of carbon nanotubes in the intracellular compartments of Kupffer cells in liver was provided by electron energy loss spectroscopy (EELS). This technique can differentiate graphitic sp<sup>2</sup> signal from CNTs and amorphous carbon signal either from the endogenous cellular background or from carbon membranes present on TEM grids (Figure 9). Moreover, EDX spectroscopy also confirmed the presence of gadolinium co-localizing with carbon nanotubes within membrane-bound intracellular compartments that are presumably lysosomes, in both liver and spleen (Figure 9). Thus, the increased MR signal in these organs is unambiguously related to the rapid uptake of CNTs by the
The massive and transient signal enhancement in bladder also suggests that a significant fraction of Gd-CNTs is eliminated by renal clearance. Both histology and TEM confirmed MRI results showing that Gd-CNTs do not accumulate in the kidneys (as only one isolated aggregate was observed in histological sections), which is consistent with effective elimination in the urine (Supporting Information Figure S7). Overall, our findings are consistent with the recent SPECT/CT imaging study on functionalized CNTs radiolabeled with $^{111}$Indium-DTPA. Different distribution profiles have been reported with a balance of renal clearance versus RES accumulation depending on the degree of chemical functionalization of the nanotubes.\footnote{16a} Individual functionalized CNTs with outer diameter lower than the glomerular filtration cut-off have been shown to escape the glomerular kidney filter to be excreted in urines in several studies.\footnote{42} In contrast, most of the largest CNTs or CNTs forming bundles in vivo are uptaken in liver, spleen and lung.\footnote{5} Therefore, full dispersion (or individualization) of CNTs which is favored by chemical functionalization appears to be a pivotal factor determining their excretion profile. In the present study, a single bolus injection of a relatively high dose of Gd-CNTs (250 µg versus 50 µg and 10 µg in two recent studies)\footnote{16a, 26} was used with no acute or sub-acute effect on the animal behavior apparent (until day 8 post-injection) and with no signs of tissue damage, inflammatory response and cellular toxicity that could be noticed on histological and TEM images in liver, spleen, kidney and lung. This clearly indicates that the dispersion of the functionalized nanotubes in physiological saline medium at the concentration of 5 mg/mL was appropriate for intravenous administration. Moreover, unlike previous studies reporting accumulation of CNTs in the lungs after their systemic administration,\footnote{5} the lung histological specimens show very few nanotubes despite the high dose injected (Supporting Information Figure S7). The lack of lung accumulation is a further indication of the good dispersibility of Gd-CNTs which persists in blood circulation. The ultrastructural examination of Gd-CNTs in liver and spleen also revealed that well individualized CNTs

---

**Figure 6.** A) $T_1$-weighted and $T_2$-weighted fast spin echo MR images of compacted cells mimicking tissue (70 µL) labeled with different concentrations (10, 20, 50 and 100 µg/mL) of Gd-CNTs or ox-CNTs. For comparison, images of agarose gel alone (agar), water alone or non-labeled cells (cells) are also displayed. $T_R/T_E$ are respectively 445/12 ms for $T_1$-weighted sequence and 4500/95 ms for $T_2$-weighted sequence. B) Longitudinal, $1/T_1$, and transverse, $1/T_2$, relaxation rates of compacted cells for different labeling conditions (NM: not measurable).
could be sequestered into intracellular compartment while keeping their native hollow and elongated structure (Figure 8). A similar intracellular distribution of CNTs was still observed 8 days after injection. This suggests that individual as well as CNT clusters were internalized in the first hours post-injection.

3. Conclusion

In conclusion, we covalently functionalized oxidized MWCNTs with DTPA for chelation of Gd\(^{3+}\) and the Gd-CNTs were administrated intravenously to mice. The CNT uptake in liver and spleen could be successfully assessed using in vivo MRI owing to the preserved capacity of Gd-CNTs to generate signal enhancement after cellular internalization. In addition, the rapid clearance into the urines of extracellular functionalized CNTs confirmed previous studies using other different imaging modalities.\(^{[43]}\) Overall, this study represents the first success in monitoring the CNT distribution with functionalized tubes used to generate a \(T_1\) positive MR contrast after intravenous injection. This work shows that combining the properties of Gd\(^{3+}\) and CNTs allows performing MRI in vivo. MRI has a high resolution compared to other imaging techniques and can thus be used as an alternative imaging method to track CNTs. Despite the fact that the amount of gadolinium chelates injected with nanotubes is far lower in clinical practice (5.75 \(\mu\)mol/kg versus 100 \(\mu\)mol/kg body weight), the high longitudinal relaxivity of Gd-CNT conjugates enabled their detection with \(T_1\)-weighting in mice using a 4.7 T scanner. In contrast to other strategies using nanoparticles to enhance MR contrast of carbon materials, the present approach exploits paramagnetic molecular chelates that are widely used in the clinics and minimally interfere with the structure, dispersibility, functionalization, loading capacity and degradability of CNTs. The approach to material design described here could open new routes to highly dispersible nanomaterials functionalizable with significant payloads for drug delivery, tumor targeting and photothermal therapy or vaccination. The second aspect is the retention of \(T_1\)-weighting properties for MRI following cellular uptake, which may provide possibilities for study the biodistribution of functionalized CNTs and for image guided delivery.

4. Experimental Section

4.1. General Information

MWCNTs (20–30 nm diameter, 0.5–2 \(\mu\)m length, 95% purity; batch 1240XH) were purchased from Nanostructured and Amorphous Materials. When stated, suspensions of CNTs were sonicated in a water bath (Transsonics Digital Elm, 20 W, 40 kHz). All reagents and solvents were purchased from different commercial suppliers and used...
as received. In particular, xylene orange tetrasodium salt was purchased from Alfa Aesar. Thermogravimetric analyses were performed by using a TGA Q500 TA instrument with a ramp of 10 °C min⁻¹ under N₂ using a flow rate of 60 mL min⁻¹. ¹H and ¹³C NMR spectra were recorded on Bruker DPX 300 instrument. The peak values were obtained as ppm (δ) and referenced to the solvent. The resonance multiplicity is indicated as s (singlet), br s (broad singlet), and m (multiplet). LC/MS analyses were performed on ThermoFisher Finnigan 6 LCQ Advantage Max. TLC was performed on aluminum sheets coated with silica gel 60 F254 (Merck, Darmstadt). Chromatographic purification was done with Merck silica gel (Kiesegel 60, 40–60 µm, 230–400 mesh ASTM). Dialysis was performed using Spectra/Por dialysis membrane, MWCO: 12–14 000 Da.

4.2. Synthesis of DTPA Ligand 2

[(2-Bromo-ethyl)-tert-butoxycarbonylmethyl-amino]-acetic acid tert-butyl ester 9: To a solution of tert-butyl bromoacetate (9.96 mL, 0.067 mol) and N,N-diisopropylethylamine (DIEA) (19.5 mL, 0.11 mol) in DMF (25 mL) was added dropwise a solution of 2-bromoethylamine hydrobromide (4.61 g, 0.022 mol) in acetonitrile (32 mL) was added a solution of H-Lys(Z)-tert-butylbromoacetate (9.96 mL, 0.067 mol) and DIEA (3.87 mL, 0.022 mol) in a phosphate buffer (Na₂HPO₄: 1.1 g/ KH₂PO₄: 0.137 g, pH 8) (36 mL). The reaction mixture was vigorously stirred at 40 °C for 3 days. Water and ethyl acetate were added and the two phases were separated. The organic phase was washed with water (twice) and brine, dried over Na₂SO₄, filtered and evaporated under vacuum. The crude was purified by chromatography on silica gel using ethyl acetate/cyclohexane from 10:90 to 70:30 as eluant.

6-Benzyloxycarbonylaminoo-2-[(bis-(tert-butoxycarbonyl)methyl-amino)-ethyl]-amino)-hexanoic acid tert-butyl ester 10: To a solution of the brominated compound 9 (6.54 g, 0.019 mol) and DIEA (3.87 mL, 0.022 mol) in acetonitrile (32 mL) was added a solution of H-Lys(Z)-OtBu·HCl (2.78 g, 0.0074 mol) in a phosphate buffer (Na₂HPO₄: 1.1 g/ KH₂PO₄: 0.137 g, pH 8) (36 mL). The reaction mixture was vigorously stirred at 40 °C for 3 days. Water and ethyl acetate were added and the two phases were separated. The organic phase was washed with water (twice) and brine, dried over Na₂SO₄, filtered and evaporated under vacuum. The crude was purified by chromatography on silica gel using ethyl acetate/cyclohexane from 20:80 to 60:40 as eluant. 3.53 g of compound 10 was obtained as yellow oil (yield: 54%). The ¹H NMR spectrum was in agreement with literature. LC-MS (ESI): m/z 745.5 [M + H]⁺.

4.3. Synthesis of Gd-DTPA Ligand 8

6-Acetylaminoo-2-[(bis-(tert-butoxycarbonyl)methyl-amino)-ethyl]-[2-(tert-butoxycarbonylmethyl-isopropoxycarbonyl)methyl-amino]-ethyl]-aminoo-hexanoic acid tert-butyl ester 6: To a solution of compound 2 (166 mg, 0.23 mmol) in methanol (2.3 mL) was added acetic anhydride (24 µL, 0.25 mmol), and the mixture was stirred at room temperature. The reaction was monitored by TLC. After 7 h, the reaction mixture was evaporated under vacuum to give 171 mg of compound 6 as a yellow
oil (yield: 98%) with a satisfactory purity. **H NMR (300 MHz, CDCl 3):** δ 6.05 (br s, 1 H), 3.41 (s, 8 H), 3.31–3.16 (m, 3 H), 2.84–2.58 (m, 8 H), 1.91 (s, 3 H), 1.64–1.22 (m, 51 H). **13 C NMR (75 MHz, CDCl 3):** 172.9, 170.8, 170.2, 81.0, 80.8, 63.9, 56.0, 53.7, 50.2, 39.4, 29.5, 29.0, 28.4, 28.3, 23.6, 23.4. LC/MS (ESI): *m/z* 787.3 [M + H] +.

6-Acetylamino-2-{bis-[2-(bis-carboxymethyl-amino)-ethyl]-amino}-hexanoic acid

A solution of compound 6 (39 mg, 0.050 mmol) in TFA (0.9 mL) and water (0.1 mL) was stirred at room temperature for 1 day. The reaction was monitored by LC/MS. The reaction mixture was precipitated in diethyl ether and filtered. The solid was dissolved in water and the solution was lyophilized to give 17 mg of compound 7 as a beige solid (yield: 68%) with a satisfactory purity. **H NMR (300 MHz, DMSO):** δ 3.35 (s, 8 H), 3.41–3.28 (m, 3 H), 3.10–2.98 (m, 8 H), 1.90–1.81 (m, 2 H), 1.79 (s, 3 H), 1.52–1.31 (m, 4 H). **13 C NMR (75 MHz, DMSO):** 172.6, 171.4, 169.1, 63.9, 54.4, 50.6, 50.3, 38.3, 29.1, 27.1, 23.6. LC/MS (ESI): *m/z* 507.3 [M + H] +.

Gd-DTPA Ligand 8: To a solution of gadolinium(III) perchlorate (Strem Chemicals, 50% in water) (14.9 µmol) in TFA (0.9 mL) and water (0.1 mL) was stirred at room temperature for 1 day. The reaction was monitored by LC/MS. The reaction mixture was precipitated in diethyl ether and filtered. The solid was dissolved in water and the solution was lyophilized to give 17 mg of compound 7 as a beige solid (yield: 68%) with a satisfactory purity. **H NMR (300 MHz, DMSO):** δ 3.35 (s, 8 H), 3.41–3.28 (m, 3 H), 3.10–2.98 (m, 8 H), 1.90–1.81 (m, 2 H), 1.79 (s, 3 H), 1.52–1.31 (m, 4 H). **13 C NMR (75 MHz, DMSO):** 172.6, 171.4, 169.1, 63.9, 54.4, 50.6, 50.3, 38.3, 29.1, 27.1, 23.6. LC/MS (ESI): *m/z* 507.3 [M + H] +.

DTPA/CNTs 4: A suspension of the functionalized CNTs 3 (21 mg) in TFA (1.8 mL) and water (200 µL) was sonicated in a water bath for 3 min and stirred at room temperature for 2 h. A solution of TFA (1.8 mL)
and water (200 µL) was added and the mixture was further stirred for 18 h. After evaporation under vacuum, diethyl ether was added and the mixture was evaporated under vacuum. The residue was dispersed in water and dialyzed against water for 2 days. The suspension was lyophilized to obtain DTPA/CNTs 4.

Gd-CNTs 5: A suspension of DTPA/CNTs 4 (8.3 mg) in gadolinium(III) perchlorate 50% aqueous solution (4.04 µmol, 8.3 mL) was sonicated for 30 min and stirred at rt for 24 h. An aliquot of the suspension was centrifuged. The supernatant was titrated with xylene orange to assess the amount of free Gd. The reaction mixture was then dialyzed against water for 2 days. The suspension was lyophilized to obtain Gd-CNTs 5.

4.5. TEM and Magnetic Characterizations of MWCNTs

Aberration-corrected High Resolution Transmission Electron Microscopy (HR-TEM), Energy-dispersive X-Ray Spectroscopy (EDX), and High Angle Annular Dark Field Scanning Transmission Electron Microscopy (HAADF-STEM): HR-TEM investigations were performed with the newly developed JEOL ARM 200 F microscope operating at 80 kV. This microscope is equipped together with a CEOS aberration corrector, a cold field emission gun, a Gatan GIF quantum ER and a JEOL EDX Diode.[43] HAADF-STEM images and EDX spectra were acquired on a FEI Tecnai G2 F20 HTREM operated at 200 kV and equipped with an EDAX super-ultra-thin window (SUWT) X-ray detector. Samples were deposited on lacey carbon Cu TEM grids (Agar).

Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) Analysis: Determination of gadolinium, iron and nickel content in CNTs was performed by ICP-AES analysis (Agilent 7500ce). The samples were digested in concentrated HNO3 (3:1, v/v) solution and diluted with ultrapure water for the analysis.

Magnetization Measurements: Magnetic measurements were carried out on a Quantum Design MPMSS-S5 SQUID Magnetometer. Field-dependent magnetization curves were measured at 5K as function of the external field in the range 0–5 Tesla. Field-cooled (FC) and zero-field-cooled (ZFC) temperature-dependent magnetization was measured using a magnetic field of 100 Oe or 1000 Oe and the sample was cooled with the same magnetic field.

NMRD and Relaxivity Measurements: The frequency dependence of the 1H relaxation for the aqueous nanotube suspensions was recorded over the frequency range 0.01–20 MHz using a Spinmaster FFC-2000 Fast Field Cycling NMR Relaxometer (Stellar SRL, Mede, Italy). T1 measurements were performed as a function of external field, B0, with standard pulse sequences incorporating B0 field excursions. The temperature of the samples was maintained at 25 ± 1 °C using a thermostated airflow system. All of the 1H magnetisation recovery curves were singly exponential within experimental error and the random errors in fitting T1 were always less than 1%. T2 was measured using the CPMG pulse sequence at 20, 40, and 60 MHz using the Stellar spectrometer and a reconditioned Bruker WP80 electromagnet. T2 was measured using the CPMG pulse sequence at 20, 40, and 60 MHz. Images of CNT liquid suspensions, agarose gel (0.3%) and cell samples (70 µL cells and 170 µL agarose) were obtained on a Bruker Bio-Spec 47/40 USR scanner operating at 4.7 T (200MHz) using a fast spin echo (TURBO RARE) sequence with a TE of 12 ms and a TR of 445 ms (T2-weighted sequence), flip angle of 180°, 30 averages, in-plane resolution of 150 µm, slice thickness of 500 nm, for a total of seven slices. At the end of the experimental protocol, mice were sacrificed with a lethal injection of sodium pentobarbital. Sivers, spleens, lungs and kidneys were excised and prepared for histological and TEM characterizations. Image processing and analysis were made with the open source software OsiriX (3.9.2, version). The percentage of contrast enhancement was defined as $SNR = \frac{\text{Mean Signal}}{\text{SD}}$, where "ref" denotes the

4.6. Cell Culture, Cell Labeling, Cell Metabolic Activity, and Cell Preparation for TEM

The murine macrophage RAW 264.7 cells were maintained as monolayer cultures in RPMI (Roswell Park Memorial Institute) medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin at 37 °C and 5% of CO2. For cell labeling, cells were incubated with complete RPMI medium supplemented with CNTs at concentration of 10, 20, 50, 100 µg/mL for 20 h at 37 °C. Cell metabolic activity was assessed by the Alamar blue test in 48-well plates seeded with cells the day before labeling. The labeled macrophages were washed twice in PBS and were incubated with 10% Alamar Blue in culture medium for 2 h. The fluorescence in cell medium due to the reduction of resazurin (oxidized form) to resorufin by cell activity was quantified on a FLUOstar OPTIMA microplate reader (excitation 550 nm, emission 590 nm) and normalized to the fluorescence signal of the control non-labeled cells. The conditions were run in quadruplicate. For TEM observation, cells were trypsinized, centrifuged at 1200 rpm for 5 min, washed in 0.1 µl cacodylate buffer and fixed with 2.5% glutaraldehyde in cacodylate buffer at 4 °C for 30 min. Cells were then post-fixed with osmium tetroxide 1% and passed through uranyl acetate. Samples were then dehydrated in an ethanol series (30–100%) and embedded in epoxy medium (EPON 812; Shell Chemical, San Francisco, California). Thin sections (70 nm) were collected onto 200 mesh cooper grids, and counterstained with lead citrate before examination with Zeiss EM902 electron microscope operated at 80 kV (MIMA2: UR1196 Génomique et Physiologie de la Lactation, INRA, Plateau de Microscopie Electronique 78352, Jouy en Josas, France).

Imaging Flow Cytometry: For imaging flow cytometry analysis, labeled macrophages were trypsinized, fixed with 4% formaldehyde in PBS and centrifuged to obtain a pellet of about 104 cells in 50 µL. Cell images were acquired using the ImageStreamX multispectral imaging flow cytometer (Amnis Corporation), collecting 10,000 events per sample at 40x magnification. Dark field images were acquired using a 785 nm laser. Cell images were analyzed using IDEAS image-analysis software (Amnis). Gating on bivariate plot of aspect ratio versus cell area was first used to isolate a population of single cells. Cells within the focal plane were further selected using a two-dimensional plot of image contrast versus root mean squared (RMS) gradient. Mean pixel feature on bright field images versus intensity feature on dark field images was used as a bivariate dot plot to discriminate cells with respect to their CNT uptake.

4.7. Animal Experiments and MRI Protocol

Animal experiments were conducted in line with French Agriculture Ministry guidelines. During the MRI protocol the animals were anesthetized with 2% isoflurane (Aerrane, Baxter, Maurepas, France) supplied in an air mixture, while their body temperature was kept constant by circulating thermostated warm water. Twelve to sixteen weeks-old female C57/B16 mice (mean weight 20.5 ± 1 g) were used in this study. The mice were imaged by the whole body MRI cell labeling, cell injection and 10, 75, 120, 360 min after injection of Gd-CNTs in the retro-orbital sinus at a dose of 1 or 5 mg/mL suspended in a 100 µL physiological saline medium. Three animals were analyzed at each time point. MRI was performed on a Biospec 47/40 USR (40-cm bore actively shielded 4.7 Tesla magnet) scanner interfaced to ParaVision software (both provided from Bruker Biospin GmbH, Rheinstetten, Germany). The whole body imaging protocol was performed with a volume transmission/reception RF coil for microwaves (Bruker), using a fast spin echo (Turbo RARE) sequence with a TE of 12 ms and a TR of 445 ms (T2-weighted sequence), flip angle of 180°, 30 averages, in-plane resolution of 150 µm, slice thickness of 500 nm, for a total of seven slices. At the end of the experimental protocol, mice were sacrificed with a lethal injection of sodium pentobarbital. Sivers, spleens, lungs and kidneys were excised and prepared for histological and TEM characterizations. Image processing and analysis were made with the open source software OsiriX (3.9.2, version). The percentage of contrast enhancement was defined as $SNR \text{post-injection} - SNR \text{pre-injection}$, where "ref" denotes the

where "SNR" is the signal-to-noise ratio.
water tube reference and SD is the standard deviation of the noise in the image. A water tube, used as a reference, was positioned near the mice to enable CNR measurement and 1H NMR signal normalization. ROIs encompassing the liver parenchyma, the whole spleen, the kidneys and the bladder were manually selected for signal measurement.

Histology: After excision, pieces of liver, spleen, lung and kidney were fixed with pH 7.4 phosphate-buffered 10% formalin and processed by embedding in paraffin. Sections, ≈6 µm thick, were evaluated after hematoxylin and eosin or Nuclear Fast Red and Pearl’s staining (all from Sigma-Aldrich, Steinheim, Germany).

Transmission Electron Microscopy in Organs: Organs were cut into 1 mm³ pieces after excision and fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixed with 1% osmium tetroxide containing 1.5% potassium cyanoferrate, gradually dehydrated in ethanol and embedded in Epon. Thin sections (70 nm) of selected zones were observed with Zeiss EM902 electron microscope operated at 80 kV as described above for cells. Ultra-thin sections (30 nm) of organs were specially prepared for high resolution imaging, EDX analysis and Electron Energy Loss Spectroscopy (EELS) analysis on the carbon K-edge at 284 eV, performed on the aberration-corrected JEOL ARM 200F operating at 80 kV.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author. TEM of ox- and Gd-CNTs, synthesis of DTPA ligand from the author. EDX in spleen.

Acknowledgements

I.M. and C.M.-M. contributed equally to this work. The authors thank C. Pécoux and S. Chat for electron microscopy, C. Fabbro for TGA measurements, O. Clément and L. Pidial for the access to the Small Animal Imaging Platform Paris-Descartes PARCC-HEGP and to the JEOL ARM 200F electron microscope recently installed at the Paris Diderot University. E.P., B.B., C.M.-M., and A.B. acknowledge the EU 7th Framework Programme (FP7-ITN Marie-Curie Network programme RADDDEL (290023). E.P. is enrolled in the UAB PhD program.

Received: July 6, 2014
Published online: September 10, 2014

[1] a) K. Kostarelos, A. Bianco, M. Prato, Nat. Nanotechnol. 2009, 4, 627; b) B. S. Wong, S. L. Yoong, A. Jagusiak, T. Panczyk, H. K. Ho, W. H. Ang, G. Pastorin, Adv. Drug Delivery Rev. 2013, 65, 1964.

[2] a) S. Lanone, P. Andujar, A. Kermanizadeh, J. Boczkowski, Adv. Drug Delivery Rev. 2013, 65, 2063; b) J. Kolosnjaj-Tabi, H. Szwarc, H. Moussa, in The Delivery of Nanoparticles, (Ed: A. A. Hashim) InTech, Rijeka, Croatia 2012.

[3] A. Bianco, K. Kostarelos, M. Prato, Chem. Commun. 2011, 47, 10182.

[4] A. Battigelli, C. Ménard-Moyon, T. Da Ros, M. Prato, A. Bianco, Adv. Drug Delivery Rev. 2013, 65, 1899.

[5] H. Ali-Boucetta, K. Kostarelos, Adv. Drug Delivery Rev. 2013, 65, 2111.

[6] a) H. Dumortier, S. Lacotte, G. Pastorin, R. Marega, W. Wu, D. Bonifazi, J.-P. Briand, M. Prato, S. Muller, A. Bianco, Nano Lett. 2006, 6, 1522; b) K. Kostarelos, L. Lacerda, G. Pastorin, W. Wu, WieckowskiSebastien, J. Luangsvilay, S. Godfroy, D. Pantarotto, J.-P. Briand, S. Muller, M. Prato, A. Bianco, Nat. Nanotechnol. 2007, 2, 108.

[7] a) C. Fabbro, H. Ali-Boucetta, T. D. Ros, K. Kostarelos, A. Bianco, M. Prato, Chem. Commun. 2012, 48, 3911; b) R. Smith, C. Zavaleta, J. Rosenberg, R. Tong, J. Ramunas, Z. Liu, H. Dai, S. S. Gambhir, Nano Today 2013, 8, 126.

[8] R. Singh, S. V. Torti, Advanced Drug Delivery Reviews 2013, 65, 2045.

[9] a) K. T. Al-Jamal, F. M. Toma, A. Yilmazer, H. Ali-Boucetta, A. Nunes, M.-A. Herrero, B. Tian, A. Eddaoudi, W. T. Al-Jamal, A. Bianco, M. Prato, K. Kostarelos, FASEB J. 2010, 24, 4354; b) K. Bates, K. Kostarelos, Adv. Drug Delivery Rev. 2013, 65, 2023.

[10] a) S. H. Ku, M. Lee, C. B. Park, Adv. Healthcare Mater. 2013, 2, 244; b) T. R. Nayak, L. Jian, L. C. Phua, H. K. Ho, Y. Ren, G. Pastorin, ACS Nano 2010, 4, 7717.

[11] a) L. Tong, Y. Liu, B. D. Dolash, Y. Jung, M. N. Slipchenko, D. E. Bergstrom, J.-X. Cheng, Nat. Nanotechnol. 2012, 7, 56; b) I. Marangon, N. Boggetto, C. Ménard-Moyon, E. Venturelli, M.-L. Béouts, C. Pécoux, N. Luciani, C. Wilhelm, A. Bianco, Nano Lett. 2012, 12, 4830.

[12] a) L. G. Delogu, G. Vidili, E. Venturelli, C. Ménard-Moyon, M. A. Zoroddu, G. Pilo, P. Nicollusi, C. Ligios, D. Bedognetti, F. Sgarrella, R. Manetti, A. Bianco, Proc. Natl. Acad. Sci. U.S.A. 2012, 109, 16612; b) A. De La Zerda, C. Zavaleta, S. Keren, S. Vaithilingam, S. Bodapati, Z. Liu, J. Levi, B. R. Smith, T-J; Ma, O. Oralkan, Z. Cheng, X. Chen, H. Dai, B. T. Khuri-Yakub, S. S. Gambhir, Nat. Nanotechnol. 2008, 3, 557.

[13] C. Zavaleta, A. de la Zerda, Z. Liu, S. Keren, Z. Cheng, M. Schipper, X. Chen, H. Dai, S. S. Gambhir, Nano Lett. 2008, 8, 2800.

[14] a) A. E. Porter, M. Gass, K. Muller, J. N. Skeep, P. A. Midgley, M. Welland, Nat. Nanotechnol. 2007, 2, 713.

[15] a) A. A. Bharde, V. Patel, J. Gavard, G. Zhang, A. A. Sousa, A. Masedunskas, R. D. Leopman, R. Weigert, J. S. Gutkind, J. F. Rulison, ACS Nano 2009, 3, 307.

[16] a) K. T. Al-Jamal, A. Nunes, L. Methven, H. Ali-Boucetta, S. Li, F. M. Toma, A. M. Herrero, W. T. Al-Jamal, H. M. M. ten Eikelder, J. Foster, S. Mather, M. Prato, A. Bianco, K. Kostarelos, Angew. Chem. Int. Ed. 2012, 51, 6389; b) J. -T. W. Wang, L. Cabana, M. Bourgognon, H. Kafa, A. Protti, K. Venner, A. M. Shah, J. K. Sosabowski, S. J. Mather, A. Roig, X. Ke, G. Van Tendeloo, R. T. M. de Rosales, G. Tobias, K. T.-Al Jamal, Adv. Funct. Mater. 2014, 24, 1880.

[17] H. Gong, R. Peng, Z. Liu, Adv. Drug Delivery Rev. 2013, 65, 1951.

[18] a) A. Al Faraj, K. Cieslar, G. Lacroix, E. Canet-Soulas, J.-P. Briand, S. Muller, M. Prato, A. Bianco, Adv. Healthcare Mater. 2012, 1, 108.

[19] V. Orazio, L. D. Suzanne, P. Andrea, C. Alfred, Nanotechnology 2011, 22, 095706.

[20] a) M. Yin, M. Wang, F. Miao, Y. Ji, Z. Tian, H. Shen, N. Jia, Carbon 2012, 50, 2162; b) G. Lamanna, A. Garofalo, G. Popa, C. Wilhelm, S. Begin-Colin, D. Felder-Flesch, A. Bianco, F. Gazeau, C. Menard-Moyon, Nanoscale 2013, 5, 4412; c) H. Wu, G. Liu, Y. Zhuang, D. Wu, H. Zhang, H. Yang, H. Hu, S. Yang, Biomaterials 2011, 32, 4867.

[21] a) L. A. Tran, M. Hernández-Rivera, A. N. Berlin, Y. Zheng, L. Sampayo, C. Bové, M. d. G. Cabrera-Hansen, J. T. Willerson, E. C. Perin, L. J. Wilson, Biomaterials 2014, 35, 720; b) Q. Ma, M. Jebb, M. F. Tweedle, L. J. Wilson, J. Mater. Chem. B 2013, 1, 5791; c) A. M. Tang, J. S. Ananta, H. Zhao, B. T. Csieros, E. Y. Lam,

Adv. Funct. Mater. 2014, 24, 7173–7186 © 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
