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**Multifunctional silica nanoparticles for optical and magnetic resonance
imaging**

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Multifunctional silica nanoparticles for optical and magnetic resonance imaging

Abstract: The surface of spherical, nonporous silica nanoparticles (SiO_2 -NPs) was modified with gadolinium (Gd) complexes, fluorophores, and cell-penetrating peptides to achieve multifunctionality on a single particle. The Gd surface concentrations were 9–16 $\mu\text{mol/g}$ resulting in nanomaterials with high local longitudinal and transversal relaxivities ($\sim 1 \times 10^5$ and $\sim 5 \times 10^5$ mm/s/NP , respectively). Rapid cellular uptake was observed *in vitro*; however, larger extracellular agglomerates were also formed. *In vivo* administration revealed a fast distribution throughout the body followed by a nearly complete disappearance of fluorescence in all organs except the lungs, liver, and spleen after 24 h. Such NPs have the potential to serve as efficient multimodal probes in molecular imaging.

Keywords: contrast agents; fluorescence imaging; magnetic resonance imaging; octaarginine; SiO_2 nanoparticles; surface modification.

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Introduction

Magnetic resonance imaging (MRI) with its high temporal and spatial resolution is currently one of the most powerful non-invasive medical diagnostic tools (Jin et al., 2009). Contrast agents (CAs) are applied to further increase the specificity and sensitivity of MRI (Ghaghada et al., 2009). The efficiency of such contrast agents is evaluated on the basis of relaxivity, i.e., the power to shorten the relaxation times of solvent water protons in their proximity. One group of CAs is engineered by chelating gadolinium (Gd) (III) ions with an organic molecule so that stable coordination complexes are formed. Their development is gaining increased attention lately because these agents require optimization to generate a maximum contrast.

Different strategies have been followed to increase relaxivity (Castelli et al., 2008; Carniato et al., 2009; Moriggi et al., 2009). One is to anchor several Gd complexes to nano-sized systems such as silica nanoparticles (SiO_2 -NPs) (Carniato et al., 2010; Henig et al., 2010; Feldmann et al., 2012; Pinho et al., 2012). The potential of such SiO_2 -NP systems for diagnosis and therapy has recently gained increased interest (Peer et al., 2007; Cho et al., 2008; Davis et al., 2008). Specific characteristics make them attractive carrier compounds for the biomedical field. First, a large payload of cargo can be attached and later released at a specific site. Second and important for MRI applications, NPs labeled with Gd generally exhibit longer *in vivo* circulation and residence times (Cheng et al., 2010b) compared to the first-generation contrast agents such as gadolinium-tetraazacyclododecane-tetraacetic acid (Gd-DOTA) and gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA) (Lowe, 2002). This might predispose them for long-term tracking applications. Third, the large surface area of NPs can be differentially functionalized and modified. However, for this multifunctionality on a single particle, facile modification of the functional surface groups followed by conjugation of the desired moieties (e.g., for imaging, treatment, or targeting) is mandatory.

In contrast to recent reports using mesoporous silica (Budny et al., 2006; Carniato et al., 2010; Cheng et al., 2010a; Vivero-Escoto et al., 2011), we report here on the use of nonporous SiO_2 -NPs to ensure that the molecules

are bound to the external surface only. As a result, there will be no hindrance for water molecules to access all Gd sites. This is mandatory for a suitable contrast enhancement. In porous materials, however, the Gd complexes are enclosed into the pores of the SiO₂ framework, thereby limiting the diffusion processes (Platas-Iglesias et al., 2002; Carniato et al., 2009). In contrast to mesoporous materials, nonporous materials have not yet been used broadly for surface functionalization. Available reports have focused mainly on attaching a single functionality (Galagudza et al., 2010). Very recently, our group reported the synthesis and characterization of monofunctionalized nonporous SiO₂-NPs for MRI applications using Gd complexes (Feldmann et al., 2012). In one case, Santra et al. (2004) have reported the coupling of additional functional groups, Tat peptide and fluorescein isothiocyanate (FITC), on SiO₂-NPs (70 nm in diameter). They first prepared a silane precursor coupled to FITC to get fluorescently labeled NPs followed by conjugation of the Tat peptide via N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) coupling chemistry.

Now, we demonstrate a facile and straightforward method to conjugate two different functionalities to obtain dual modal probes, both for optical and MR imaging. Apart from being performed easily, it allows for a higher flexibility in exchanging the functional moieties for different applications. The exterior surface of these NPs was functionalized by a selective protection-deprotection strategy allowing to conjugate various moieties. As our group is involved in the design and synthesis of targeted contrast agents (Joshi et al., 2010; Mishra et al., 2012), the anchoring moieties, herein, were chosen to study the cellular uptake and contrast enhancement ability in MR images. Furthermore, we aimed to demonstrate the multifunctionalization and applicability of the thus obtained SiO₂-NP. We conjugated Gd-DO3A hexylamine (Gd-DO3A-HA) as an MRI contrast agent, a cell-penetrating peptide (octaarginine, R₈) to enhance the cellular uptake, and/or fluorophores for optical imaging. We describe the chemistry of surface modification, MRI contrast enhancement, cellular uptake and localization, and finally, the initial results for the biodistribution *in vivo*. We comparatively assessed two types of SiO₂-NP differing in the chain length of the alkyl spacer (C₄ or C₁₁) attached on the surface.

Results and discussion

Functionalization of the nanoparticles

Spherical, nonporous, and monodisperse SiO₂-NP, with an average diameter of about 100 nm, were synthesized

by the Stöber process (Stöber et al., 1968; Plumere et al., 2012), and the exterior surface was further tailored by covalent bonding. In the first step, the surface of the bare SiO₂-NP was functionalized with Si-H bonds (Plumere et al., 2009). Next, it was modified with carboxylic acid residues by a photochemically induced hydrosilylation reaction of 10-undecylenic acid (Plumere and Speiser, 2007) and 3-butanolic acid resulting in MC_{n+2}COOH (where n=1 or 8, Feldmann et al., 2012). The synthesis of this precursor material and its full characterization by dynamic light scattering (DLS), scanning electron microscopy (SEM), diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy are described in our recent publication (Feldmann et al., 2012). The biprotected lysine derivative N_α-Fmoc-N_ε-Dde-D-Lys was used as a bifunctional linker. First, the Fmoc protecting group was cleaved, and the side chain-protected lysine derivative was coupled to both types of particles (C₄ and C₁₁), producing SiO₂-NP connected to the lysine linkers through a peptide bond (Figure 1). Thus, the exterior surface became bifunctional, namely, with 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) protected amines and free carboxylic acids. In the next step, the carboxylic acid groups were reacted with the macrocyclic Gd complex (Gd-DO3A-HA) to serve as CA for MRI. The deprotection of the Dde group with 2% hydrazine in dimethylformamide (DMF) yielded amine termini, which were covalently coupled to either FITC or Cy 5.5 alone or to a lysine-FITC-labeled octaarginine (R₈). Thus, the particles were modified to serve for bimodal (magnetic resonance and optical) imaging. Activation, as well as coupling reactions, was done by a standard peptide synthesis procedure. The removal of the remaining reactants and activators was achieved by repeated washes using centrifugation. The successful coupling of the fluorophore or fluorescently labeled peptides to the NPs was confirmed by polyacrylamide gel electrophoresis (PAGE). Only the distinct fluorescent bands of the fluorescently labeled particles were seen directly on top of the gels (data not shown).

Size of the nanoparticles

The size distribution and agglomeration behavior of the particles were evaluated by dynamic light scattering (DLS) in phosphate-buffered solution (PBS, Table 1). The spacer length had a significant influence on the average hydrodynamic diameter: NPs with the long C₁₁ chain linker [MC₁₁-K(FITC)-Gd and MC₁₁-KR₈K(FITC)-Gd] were about 1.5 to 1.7 times larger than with the short spacer

Figure 1 Synthesis of modified SiO₂-NPs.

The chemical structures of the molecules used for the surface modification are shown below the reaction scheme.

[$MC_4-K(FITC)-Gd$ and $MC_4-KR_8K(FITC)-Gd$]. However, the coupling of the highly positively charged octaarginine had no additional influence on the hydrodynamic diameter. In contrast, an even smaller average diameter of about 123 nm was observed for the R_8 -conjugated C_4 material compared to the corresponding NPs with fluorophore alone (~141 nm).

Surface concentration of gadolinium

The concentration of carboxylic acids on the starting materials MC_4COOH and $MC_{11}COOH$ were 640 $\mu\text{mol/g}$ and 150 $\mu\text{mol/g}$, respectively, as quantified by elemental analysis and thermogravimetric analysis as described in Feldmann et al. (2012). It is evident that steric constraints permit to

MC ₄ -K(FITC)-Gd	MC ₄ -KR ₈ K(FITC)-Gd	MC ₁₁ -K(FITC)-Gd	MC ₁₁ -KR ₈ K(FITC)-Gd
141±3***	123±4***	212±21	210±2

Table 1 Hydrodynamic diameter (in nm) of the four synthesized materials.

Measured in PBS buffer at 25°C with the Zetasizer Nano ZS from Malvern Instruments. Samples were filtered prior to the measurements. Values represent means±SEM (n=4). ****p*<0.001, significantly different compared to MC₁₁-K(FITC)-Gd and MC₁₁-KR₈K(FITC)-Gd.

functionalize only a low percentage of the reacting sites on the surface for both types of spacers. After lysine coupling followed by conjugation of Gd-DO3A-HA, T₁-measurements yielded surface Gd concentration of 21 μmol/g (MC₄-K-Dde-Gd) and 11 μmol/g (MC₁₁-K-Dde-Gd). Further functionalization with FITC or R₈-FITC slightly decreased the Gd content to about 16 μmol/g for both MC₄-K(FITC)-Gd and MC₄-KR₈K(FITC)-Gd, as well as 11 and 9 μmol/g for MC₁₁-K(FITC)-Gd and MC₁₁-KR₈K(FITC)-Gd, respectively, due to the increase in particle weight.

For further experiments, stock dispersions of SiO₂-NPs in water were prepared at 10 mg/ml. These stocks were thoroughly mixed and sonicated prior to the preparation of the sample dispersions at concentrations indicated below.

In vitro cell studies

The cellular uptake and localization of the SiO₂-NPs were studied by fluorescence spectroscopy and microscopy. The fluorescently labeled particles [MC₄-K(FITC)-Gd, MC₄-KR₈K(FITC)-Gd, MC₁₁-K(FITC)-Gd, and MC₁₁-KR₈K(FITC)-Gd] were dispersed in serum-containing cell culture medium at different concentrations and were added onto 3T3 mouse fibroblasts for 18 h. Fluorescence spectroscopy demonstrated that these particles were accumulating in (or on) cells in a concentration-dependent manner (Figure 2A). The conjugation of the fluorescently labeled octaarginine increased the measured fluorescence about 3.5 times compared to the solely FITC-labeled materials. This observation could be made even after quenching of the extracellular fluorescence by Trypan Blue (Rennert et al., 2006). Fluorescence microscopy, however, revealed that large fluorescent aggregates (in parts >1 μm) were formed in particular for the R₈-containing SiO₂-NPs that remained in the extracellular space after washing of the cells and whose fluorescence could not be completely quenched (Figure 2B–F). The yellow arrows point to the large extracellular agglomerates, and the white arrows indicate the smaller predominantly intracellular vesicles filled with NPs in the perinuclear region of the cells (Figure 2C–F). This finding of cellular uptake is analog to recently published reports (Al-Rawi et al., 2011; Quignard

et al., 2012), where similar NPs were efficiently internalized. We found that the NPs with a short alkyl chain on the surface [MC₄-K(FITC)-Gd, MC₄-KR₈K(FITC)-Gd] were slightly better internalized than the materials with long alkyl chains [MC₁₁-K(FITC)-Gd and MC₁₁-KR₈K(FITC)-Gd]. However, due to the observed extracellular agglomeration and the incomplete quenching of the fluorescence, the spectroscopic results should be taken with caution.

Because the C₄ particles had a higher loading of the Gd complexes and were slightly better internalized into the cells, only these materials were used for further experiments.

Magnetic resonance imaging studies

All the MRI experiments were performed in 1.5% agar to stabilize the dispersion of the materials throughout the measurement. Agar pellets (100 μl) were prepared in the respective solvent (water, pH=7, or serum-containing cell culture medium). The longitudinal and transverse relaxation rates were evaluated, and sagittal as well as axial T₁-weighted images were made in a clinical MR scanner at 3T and room temperature. There was a concentration-dependent increase of the longitudinal relaxation rate R₁ for MC₄-K(FITC)-Gd and MC₄-KR₈K(FITC)-Gd as shown for a representative experiment in Figure 3A. The concentration of Gd on the particles (and thus inside the dispersion) was determined by T₁ measurements of acid-hydrolyzed SiO₂-NPs and Gd standard samples as described elsewhere (Feldmann et al., 2012). The calculated longitudinal relaxivity r₁ of the NPs is given in Table 2. The r₁ (per Gd) of MC₄-K(FITC)-Gd and MC₄-KR₈K(FITC)-Gd in water was 8.7 /mM/s and 6.9 /mM/s, respectively, whereas 5.2 /mM/s and 3.5 /mM/s, respectively, were obtained in cell culture medium. Thus, coupling of the Gd-DO3A-HA complex to the surface of the SiO₂-NPs slightly increased r₁ for MC₄-K(FITC)-Gd (+53% and +73% in water and medium, respectively) and MC₄-KR₈K(FITC)-Gd (+21% and +17% in water and medium, respectively) compared to the free complex (5.7 /mM/s and 3.0 /mM/s in water and medium, respectively) (Feldmann et al., 2012).

The lower r₁ values in the medium compared to water are most likely due to the presence of anions such as

Figure 2 Cellular uptake of SiO₂-NPs in 3T3 mouse fibroblasts *in vitro*.

(A) Relative quantification of uptake/binding (fluorescence spectroscopy). The cells were incubated at indicated concentrations of SiO₂-NPs for 18 h in 96-well plates. The cell nuclei were counterstained with the DNA dye Bisbenzimidazole Hoechst 33342, the extracellular fluorescence was quenched by Trypan Blue, and the cells were thoroughly washed with HBSS prior to measurement. The green fluorescence of FITC-labeled SiO₂-NPs and the blue fluorescence of Hoechst 33342 (as measure of cell number) were used to determine the cell number-normalized fluorescence. (B) The characteristic morphological changes of the cells in contact with large agglomerates of SiO₂-NPs [here 25 μg/ml MC₄-KR₈K(FITC)-Gd, 18 h]. The same cells in 96-well plates were examined by microscopy. The overlay of phase contrast, green (SiO₂-NPs), and blue (cell nuclei) channel. The agglomerates were surrounded by cellular membrane structures but were not internalized. The bar represents 5 μm. (C–F) High-resolution phase contrast (C, D) and corresponding fluorescence images (E, F) of 3T3 mouse fibroblast cells after the incubation with SiO₂-NPs. The cells were labeled for 18 h with SiO₂-NPs (C, E) MC₄-K(FITC)-Gd and (D, F) MC₄-KR₈K(FITC)-Gd in cell culture medium (33 μg NP/ml, green fluorescence). The cell nuclei were counterstained (Hoechst 33342, blue), and the extracellular fluorescence was quenched by Trypan Blue (red fluorescence) as described above. The yellow arrows point to the large agglomerates clearly located outside the cells, the white arrows to small intracellular internalized SiO₂-NPs. The bars represent 20 μm.

CO₃²⁻ and PO₄³⁻ in the medium and their interaction with Gd in the chelates of the DO3A-like structure, which leads to the displacement of Gd-bound water molecules and in consequence to a reduction of the observed relaxivity (Caravan et al., 1999; Dickins et al., 2002).

The obtained r_1 values were only slightly increased compared to the free low molecular weight Gd-DO3A-HA and were similar as for the recently reported monofunctionalized Gd-loaded SiO₂-NPs (Feldmann et al., 2012). Thus, the additional coupling of a fluorophore or even of a short peptide is not significantly influencing the high local rotational dynamics of the hexyl amine-linked

DO3A-ligand, which diminished the increase in relaxivity due to the slow global tumbling of the whole particle.

In contrast, the transverse relaxivity r_2 of both MC₄-K(FITC)-Gd (42.3/mm/s and 56.8/mm/s in water and medium, respectively) and MC₄-KR₈K(FITC)-Gd (46.5/mm/s and 46.1/mm/s) was much larger compared to the free Gd-DO3A-HA (12.0/mm/s and 5.1/mm/s, respectively).

It can be concluded that immobilization of Gd-DO3A-HA on the particle surface significantly increased r_2 , whereas r_1 is less influenced at a field strength of 3T.

Even though the longitudinal relaxivity r_1 per Gd of the material is only slightly increased, the high number

Figure 3 Magnetic resonance imaging of SiO₂-NPs.

(A) The longitudinal relaxation rate R_1 in agar phantoms with various concentrations of MC₄-K(FITC)-Gd and MC₄-KR₈K(FITC)-Gd (one representative experiment). (B) The corresponding axial T_1 -weighted MR images of MC₄-K(FITC)-Gd and MC₄-KR₈K(FITC)-Gd in aqueous agar phantoms. The given concentrations are 50–400 μg NPs/100 μl agar solution (corresponding to 8–64 μM of Gd).

of Gd per particle led to the high relaxivities per particle for both MC₄-K(FITC)-Gd ($r_1=1.1 \times 10^5$ /mm/s and $r_2=5.2 \times 10^5$ /mm/s) and MC₄-KR₈K(FITC)-Gd ($r_1=0.8 \times 10^5$ /mm/s and $r_2=5.6 \times 10^5$ /mm/s) (Table 2). Thus, high local relaxivities can be achieved for these non-porous SiO₂-NPs with multiple functionalities.

The dispersed particles showed a clear contrast enhancement of the agar layer in T_1 -weighted MR images (Figure 3B). Samples of MC₄-K(FITC)-Gd and MC₄-KR₈K(FITC)-Gd containing more than 50 μg (dispersed in 100 μl) in water and 100 μg (dispersed in 100 μl) in medium displayed significantly increased signal

intensities. A voxelwise analysis of the signal intensities for these images revealed a significant difference for all the concentrations and types of the NPs compared to the control (data not shown).

Biodistribution studies

The biodistribution and mobility of the NPs play an important role for their *in vivo* applicability. To assess the biodistribution, FITC was replaced with the near-infrared dye Cy 5.5 for a better detectability by the optical methods.

	Number of Gd/NP ($\times 10^4$)	Fluid	Relaxivity per mM Gd (/mm/s)		Relaxivity per mM NP (/mm/s)	
			r_1	r_2	$r_1 (\times 10^5)$	$r_2 (\times 10^5)$
MC ₄ -K(FITC)-Gd	1.23	Water	8.7±0.4	42.3±3.7	1.1±0.1	5.2±0.5
		Medium	5.2±0.4	56.8±2.2	0.6±0.1	7.0±0.3
MC ₄ -KR ₈ K(FITC)-Gd	1.20	Water	6.9±0.7	46.5±9.7	0.8±0.1	5.6±1.2
		Medium	3.5±1.0	46.1±6.7	0.4±0.1	5.5±0.8

Table 2 Longitudinal and transversal relaxivities of the functionalized materials in agar phantoms of water and culture medium.

Values represent mean±SEM (n=4–8). MRI measurements and determination of the Gd surface concentration of the materials were performed as described under the Materials and methods section and in Feldmann et al. (2012). The relaxivities per Gd were determined from the slope of the linear fit of the observed relaxation rates vs. the Gd concentration. Given the Gd surface concentration, the number of Gd per particle and, thus, the relaxivity per particle was calculated.

The Cy 5.5-coupled NPs [MC₄-K(Cy 5.5)-Gd] were injected intravenously into mice, the animals were sacrificed at 20 s, 10 min or 24 h later, and *ex vivo* optical imaging of the derived organs was performed (Figure 4A, displaying results at 20 s and 14 h). The particles were readily transported via the blood stream. A strong fluorescence signal was observed in the lungs already within 20 s after injection. Lower fluorescence intensity was also seen in the liver, kidneys, spleen, heart, and brain, but not in the intestine. At 24 h, the signal intensity was reduced in all the organs. However, a still elevated level of fluorescence was observed in the lung, liver, and spleen (Figure 4B) indicating that the used NPs persistently accumulated in these organs. The liver and kidney were subsequently analyzed using fluorescence microscopy. The Cy 5.5-coupled NPs were quantified by counting the fluorescent particles within the frozen tissue sections. Quantification in the histological sections confirmed the results obtained with

optical imaging. In the liver sections, 231 ± 108 ($n=3$) particles were counted at 10 min after injection, whereas their number was reduced to 55 ± 24 ($n=17$) after 24 h. The kidney sections contained significantly less particles (22 ± 4 , $n=5$) at 10 min. After 24 h, only a few particles could be detected (2 ± 1 , $n=16$). Figure 4C displays a representative microscopic image of a liver slice with several fluorescent particles visible (yellow arrows). Furthermore, the histological evaluation revealed an aggregate formation within the liver with a mean size of about 1 μm (Figure 4D). These findings confirmed the already *in vitro* observed tendency of the materials to form large agglomerates *in vivo* as well. Of note, no signs of toxicity could be documented during the whole observational period.

Because of the lack of suitable tracking methods, the fate of SiO₂-NPs after intravenous injection has not been extensively reported yet. However, Cho et al. (2009) have compared the tissue distribution and excretion of

Figure 4 Biodistribution of MC₄-K(Cy 5.5)-Gd.

The SiO₂-NPs were injected intravenously into BALB/c mice (0.3 mg/200 μl /20 g body weight). The animals were sacrificed after 20 s, 10 min, or 24 h later, and optical imaging of the *ex vivo* derived organs was performed. (A) Fluorescence imaging of organs 20 s or 24 h after intravenous injection. The control animal received the same volume of buffer without the NPs. The images were obtained on a Maestro™ 2 system for *in vivo* optical imaging. A spectral unmixing algorithm was used to subtract the background autofluorescence. (B) The biodistribution analysis by fluorescence intensity of the *ex vivo* images. The signal intensities were evaluated using ImageJ software (<http://rsb.info.nih.gov/ij>) by defining a circular region of interest (ROI, 274 pixels) inside the respective organs. The values represent mean $\pm$ SD. (C) The representative fluorescence microscopy image of a liver slice. The yellow arrows point to the violet-pink fluorescence of the SiO₂-NPs. The autofluorescence of the liver tissue is depicted in blue. (D) The particle size measured in the liver 10 min postinjection (fluorescence microscopy).

different-sized NPs (50, 100, and 200 nm in diameter) in mice. It was reported that the distribution as well as the excretion depended on the particle size. The excretion took place via the urine and feces and was fastest for the smallest particles (50 nm). The larger the SiO₂-NPs, the more they were trapped in the spleen and the liver after the uptake by macrophages and remained in these organs over 4 weeks. In another report, Xie et al. (2010) found as well that SiO₂-NPs (diameters of 20 and 80 nm) were taken up mainly by the macrophages of the reticuloendothelial system and accumulated in the liver and spleen where they could be detected even at day 30 postinjection. However, in a similar study on the clearance of smaller-sized SiO₂-NPs (20–25 nm), Kumar et al. (2010) have shown that such NPs can be effectively excreted via the hepatobiliary system in a few hours. In this context, the recent findings of Quignard et al. (2012) can be of high interest where a slow degradation of the SiO₂-backbone of nonporous NPs to small soluble SiO₂-species was observed *in vitro* in human fibroblasts.

In conclusion, a simple and straightforward method to obtain a multifunctional surface on a spherical and nonporous SiO₂ support was presented allowing the preparation of the bimodal NPs for optical and MR imaging. Out of the two alkyl chain spacers (C₄ and C₁₁) attached on the surface of the SiO₂-NPs, the shorter was found to be more suitable for obtaining a high Gd surface load. A high relaxivity per particle was achieved that ensures the potential of the material to be an efficient contrast agent for MRI applications. Our results demonstrated that the particles could be taken up by 3T3 mouse fibroblasts. However, the NPs tend to form larger agglomerates in a biological environment, which then cannot be internalized by the cultured cells *in vitro* anymore. Intravenously injected NPs were initially distributed to almost all organs (except the gut); however, they appeared mainly in the lungs. After 24 h, a sustained accumulation was only observed in the lung, liver, and, to a smaller extent, in the spleen, whereas the fluorescence dropped to control levels in all the other organs.

The potential of such SiO₂-NPs to be used as bimodal probes for optical and MR imaging could be shown. In order to further increase the cellular uptake as well as the cytosolic concentration, coupling of polyethylene glycol (PEG) to the particles can help as shown by Cheng et al. (2010a) for microparticles. However, some important drawbacks have to be resolved in the future such as the agglomeration and long-term retention in the body with its subsequent implication for toxicity. To further the specificity of such molecular imaging modalities, it will be needed to attach targeting moieties as well. Currently, we aim to couple antibodies against cellular surface

structures of interest to the Gd complex/Cy 5.5 NPs for such specific targeting approaches. In addition, it is possible to incorporate chemotherapeutic drugs (e.g. Camptothecin or Bortezomib) on the surface of our SiO₂-NPs after some modifications. In combination with the respective targeting moieties, these drugs can be selectively delivered to the diseased cells or tissue. Thus, these materials have the potential to be useful in the diagnosis and therapy as well. Our current results are promising as they indicate that nonporous SiO₂-NPs with multiple functionalities offer a platform for the development of highly efficient multimodal and/or targeted nano-sized probes for molecular imaging and therapeutic applications.

Materials and methods

General

All chemicals were obtained from commercial sources and used without further purification. Trifluoroacetic acid (TFA), DMF, dichloromethane (DCM), *N,N*-diisopropylethylamine (DIEA), triisopropylsilane (TIPS), FITC, 1-hydroxybenzotriazole (HOBt), and *N,N'*-dicyclohexylcarbodiimide (DCC) were obtained from Acros Organics (Belgium). Gd chloride was purchased from Sigma-Aldrich (Germany). Cy-5.5 NHS ester was purchased from Lumiprobe (USA). The hydrazine hydrate solution was obtained from Fischer Scientific (Germany). O-(7-Azabenzotriazol-1-yl)-*N,N,N'*-tetramethyluronium hexafluorophosphate (HATU) and O-(Benzotriazol-1-yl)-*N,N,N'*-tetramethyluroniumtetrafluoroborate (TBTU) were purchased from Applied Biosystems (Germany) and Merck (Germany), respectively. Protected Fmoc-amino acids as well as resin were obtained from Novabiochem (Germany).

For the analysis and purification of CH₃-CO-Lys(FITC)-R₈-COOH, analytical and semipreparative RP-HPLC was performed at room temperature on a Varian PrepStar instrument (Australia) equipped with PrepStar SD-1 pump heads. UV absorbance was measured using a ProStar 335 photodiode array detector at 214 nm. A Varian Polaris C18-Ether column (4.6×250 mm, particle size 5 μm, pore diameter 100 Å) was used for analytical RP-HPLC. For the semipreparative HPLC, a Varian Polaris C18-Ether column (21.2×250 mm, 5 μm, 100 Å) was used. The method used for HPLC was as follows. A linear gradient was used starting from 80% solvent A (0.1% TFA in water) and 20% of solvent B (0.1% TFA in acetonitrile) to 90% B in 30 min (flow rate of 1 ml/min for analytical and 3 ml/min for semipreparative HPLC).

Electrospray ionization mass spectrometry (ESI-MS) was performed on Agilent SL 1100 Series LC/MSD Trap system (Agilent, Germany): nebulizer, 20.0 psi; dry gas, 5.0 l/min; dry temperature, 250°C.

Synthesis of modified silica nanoparticles

The synthesis of carboxylic acid-functionalized SiO₂-NPs, MC_{n-2}COOH, was performed as described in our earlier publication

(Feldmann et al., 2012). Lysine-Dde prepared by treating Fmoc-Lys (Dde)-OH with 20% piperidine in DMF (10 min, 2×) and subsequent evaporation on the rotary evaporator was reacted with MC_{n+2}COOH (TBTU, DIPEA, 2 h, RT) to produce MC_{n+3}-K-Dde. The carboxylic acid part of it was activated and further reacted with Gd-DO3A-HA under the above-mentioned reactions conditions. After that, the Dde-protecting group was cleaved by 2% hydrazine in DMF (2.5 min, 2×) to afford a free amino group, which was either coupled to FITC or Cy 5.5 by mixing the fluorophores and DIPEA with NPs for 20 h or to CH₃-CO-Lys(FITC)-R₈-COOH in the presence of HATU, DIPEA, 20 h. CH₃-CO-Lys(FITC)-R₈-COOH was synthesized on Wang resin by solid-phase peptide synthesis with Fmoc/tBu-strategy on an automatic synthesizer (Prelude™ peptide synthesizer, Germany) and purified similar to our already published protocol (Joshi et al., 2011). The detected molecular ions at $m/z=913.9$ [(M+2H)²⁺], 609.8 [(M+3H)³⁺] were consistent with the calculated mass of CH₃-CO-Lys(FITC)-R₈-COOH 1826.0.

Synthesis of Gd-DO3A-HA

The synthesis of Gd-DO3A-HA was performed as reported elsewhere (Feldmann et al., 2012).

Size of nanoparticles

The hydrodynamic diameter of the four materials was measured in PBS buffer at 25°C with the Zetasizer Nano ZS from Malvern Instruments (Germany). The samples were filtered prior to the measurements.

Cell culture

The 3T3 NIH mouse fibroblasts were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin (all purchased from Biochrom AG, Germany) at 37°C, 10% CO₂. The cells were trypsinized and passaged twice a week.

Cellular uptake by fluorescence spectroscopy

The uptake efficiency was assessed using 3T3 fibroblasts grown to 70–80% confluency in 96-well microplates. The cells were incubated at indicated concentrations of SiO₂-NPs in complete culture medium for an additional 18 h at 37°C, 10% CO₂. The cell nuclei were counterstained with the DNA dye Bisbenzimidazole Hoechst 33342 (Sigma-Aldrich, Germany) for 30 min, the extracellular fluorescence was quenched by Trypan Blue (Rennert et al., 2006), and the cells were thoroughly washed with Hank's buffered saline (HBSS) prior to measurement in a multiplate reader (BMG Labtech, Germany). The green fluorescence of FITC-labeled SiO₂-NPs (Ex 485 nm/Em 530 nm) and the blue fluorescence of Hoechst 33342 (Ex 345 nm/Em 460 nm; as measure of cell number) were used to determine the cell number-normalized fluorescence.

Intracellular localization by microscopy

The same cells in the 96-well microplates, used before for spectroscopy, were imaged without fixation using a Zeiss Axiovert 200 M microscope (Zeiss, Germany) with a LD Plan NeoFluor 40× objective.

High-resolution microscopic images were obtained by incubating 3T3 mouse fibroblast cells with SiO₂-NPs in µ-Dishes (Ibidi, Germany) and using a Plan-Apochromat 63× objective. The cells were labeled for 18 h with SiO₂-NPs [MC₄-K(FITC)-Gd and MC₄-KR₈K(FITC)-Gd] in the cell culture medium (33 µg SiO₂-NP/ml). The cell nuclei were counterstained (Hoechst 33342), and extracellular fluorescence was quenched by Trypan Blue as described above.

Gd content from T₁ measurements

The Gd content of functionalized SiO₂-NPs was estimated from T₁-measurements. In brief, the Gd-functionalized particles were suspended in concentrated HNO₃ and heated to 120°C for 24 h to mineralize the samples and release the Gd from the chelates. Standard GdCl₃ solutions (0.01–2 mM) were prepared and comparable amounts of unfunctionalized SiO₂-NPs were added and treated with HNO₃, like for functionalized particles. The Gd concentrations were determined from a standard curve following the equations as reported in detail in our earlier publication (Feldmann et al., 2012).

MR imaging of silica nanoparticles in agar phantoms

The MRI experiments were performed in 1.5% agar to stabilize the dispersion of the material throughout the measurement. The stock dispersions were diluted in water or cell culture medium and mixed with the same volume of heated 3% agar (in water or medium) to obtain 1.5% agar dispersion at the indicated concentrations of SiO₂-NPs. Of these dispersions, 100 µl was filled into 0.625-ml reaction tubes, were allowed to gel, and afterwards overlaid with 400 µl of the corresponding solvent. These samples were imaged at 3T (123 MHz) and room temperature in a Siemens TIM Trio clinical scanner (Germany), and the relaxation rates were determined as described elsewhere (Feldmann et al., 2012). The longitudinal relaxation times (T₁) were measured using an inversion recovery sequence to obtain images from an axial slice of 1 mm thickness through the samples. The inversion time (Ti) was varied from 23 ms to 3000 ms in 12 steps. The images were read out with a turbo spin echo technique, acquiring five echoes per scan. The repetition time (TR) was 10 000 ms to ensure complete relaxation. Six averages per Ti were possible within 18 min. For T₂, a home-written spin-echo sequence was used with the echo times varying from 25 ms to 275 ms in 10 steps and a repetition time of 8 s. The diffusion sensitivity was reduced by minimizing the crusher gradients surrounding the refocusing pulse. A matrix of 256×256 voxels was used over a field-of-view of 110×110 mm² resulting in a voxel volume of 0.43×0.43×1 mm³.

The data analysis was performed by fitting of the relaxation curves with the self-written routines under MATLAB 7.1 R14 (The Mathworks Inc., USA). The series of T₁ and T₂ relaxation data were fitted to the equations 1 and 2.

$$T_1 \text{ series with varying } t = T_i: S = S_0 [1 - \exp(-t/T_1)] + S_{(Ti=0)} \exp(-t/T_1) \quad (1)$$

$$T_2 \text{ series with varying } t = T_e: S = S_0 \exp(-t/T_2) \quad (2)$$

The nonlinear least-squares fitting of three parameters S_0 , $S_{(Ti=0)}$, and T_1/T_2 was done for the manually selected regions of interest with the Trust-Region Reflective Newton algorithm implemented in MATLAB. The quality of the fit was controlled by visual inspection and by calculating the mean errors and residuals. The obtained T_1/T_2 values of the samples were converted to $R_1 (=1/T_1)$ and $R_2 (=1/T_2)$.

The relaxivities per Gd were determined from the slope of the linear fit of the observed relaxation rates vs. the Gd concentration. Given the Gd surface concentration, the number of Gd per particle and, thus, the relaxivity per particle was calculated.

Biodistribution of MC₄-K(Cy 5.5)-Gd

The SiO₂-NPs were injected intravenously into BALB/c mice (0.3 mg/200 µl/20 g body weight). The animals were sacrificed after 20 s, 10 min, or 24 h later, and optical imaging of the *ex vivo* derived

organs was performed. The control animal received the same volume of buffer without the NPs. The images were obtained on a Maestro™ 2 system for *in vivo* optical imaging (Caliper Life Sciences, Germany). A spectral unmixing algorithm was used to subtract the background autofluorescence. For the biodistribution analysis, the fluorescence signal intensities were evaluated using ImageJ software (<http://rsb.info.nih.gov/ij>) by defining a circular region of interest (ROI, 274 pixels) inside the respective organs. Afterwards, frozen tissue sections were made from the liver and kidney samples at 10 min and 24 h postinjection. The histological sections were analyzed by fluorescence microscopy. The NPs/agglomerates were counted, and their mean size was determined as well.

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