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A Novel Method of Cellular Labeling: Anchoring MR-Imaging Reporter Particles on the Outer Cell Surface

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Cell labeling has been receiving increased attention for its implications in imaging-guided cellular therapies. The superb anatomical resolution attainable in MR images makes MRI the technique of choice for in vivo cell tracking, assessment of the fate of transplanted cells, and for the evaluation of their therapeutic effects.[1] Currently cellular labeling for MRI applications consists of cell entrapment of Gd-containing contrast agents[2–4] or of iron-oxide particles,[5–7] the latter being the more favored approach. Both labeling procedures involve the internalization of the imaging reporting units into endosomal vesicles. To be MRI-detectable a cell has to be loaded with a number of metal atoms in the order of 10^8–10^{10} per cell.[8] Although the endosomal entrapment appears to limit the toxicity risks associated with the uptake of such a huge number of metal atoms, concern exists on the long-term metabolic fate of the internalized systems. Actually, internalization procedures are well established methods in nuclear medicine and the methodologies used for MR-labeling may be envisaged as a simple extension of those methods. However the huge difference in the number of imaging reporter units needed in the two modalities call for caution when one has to deal with such a huge payload as required for MR detection.

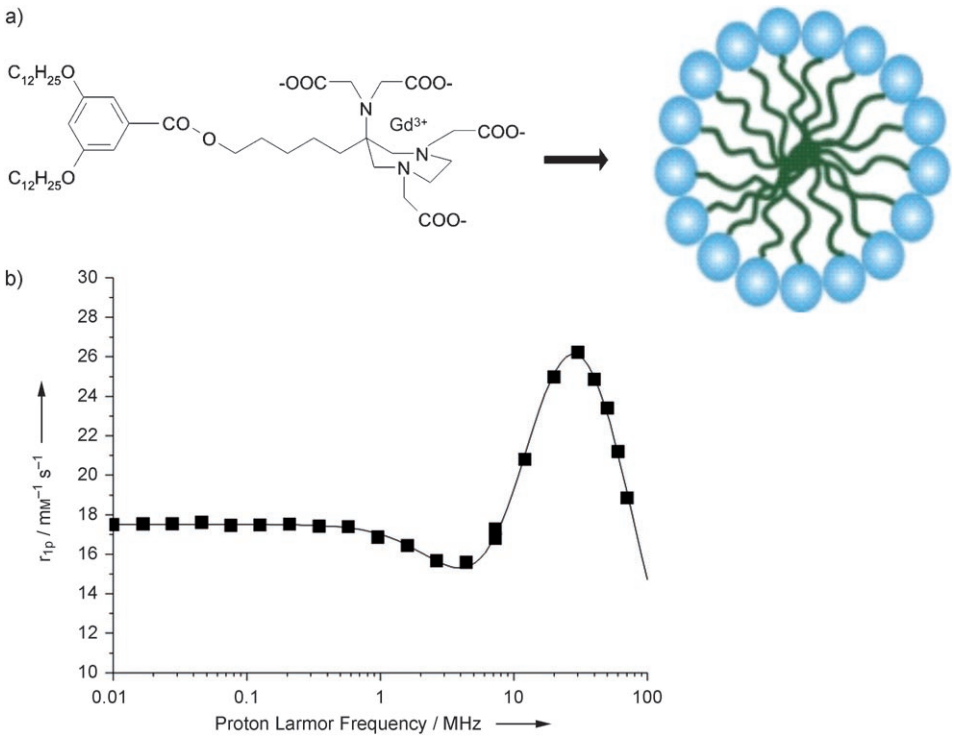

Figure 1. The design of the high sensitivity MR imaging reporter. a) Self-assembly of lipophilic Gd^{III}-chelates yields nanosized supramolecular structures (mean diameter 80 nm) containing approximately 800 paramagnetic centers. b) $1/T_1$ nuclear magnetic relaxation dispersion (NMRD) profile of the Gd-particles measured at 25 °C and neutral pH, normalized to 1 mM concentration of Gd^{III} ion. The solid curves through the data points were calculated using the Solomon-Bloembergen equations according to the Lipari-Szabo approach. The obtained τ_l (associated to the local mobility) and τ_g (associated to the overall tumbling) values were 507 ps and 2690 ps, respectively (order parameter $S^2 = 0.56$). The distance between Gd^{III} ion and the protons of the coordinated water molecule was fixed at 3.1 Å, whereas the distance between Gd^{III} ion and the outer sphere water proton nuclei at 3.8 Å; the solute-solvent diffusion coefficient was fixed at 2.24×10^{-5} $cm^2 s^{-1}$. The exchange lifetime (τ_M^{298}) of the coordinated water molecule, was determined by ^{17}O-NMR studies (145 ns, data not shown).

An alternative approach for cellular labeling would be linking the imaging reporters on the cell surface. One may think of covalent or noncovalent approaches. Herein we report a proof of concept in which self-assembled Gd-complexes are linked in a noncovalent way to the cell surface.

The idea is to anchor particles containing a high number of paramagnetic Gd^{III}-complexes at the cell surface without compromising the overall characteristics of the cell membrane. The paramagnetic particles consist of self-assembled lipophilic Gd–AAZTA complexes (Figure 1a—see Supporting Information for their synthesis). The presence of two long aliphatic chains and the aromatic moiety endow this anphiphilic complex with a

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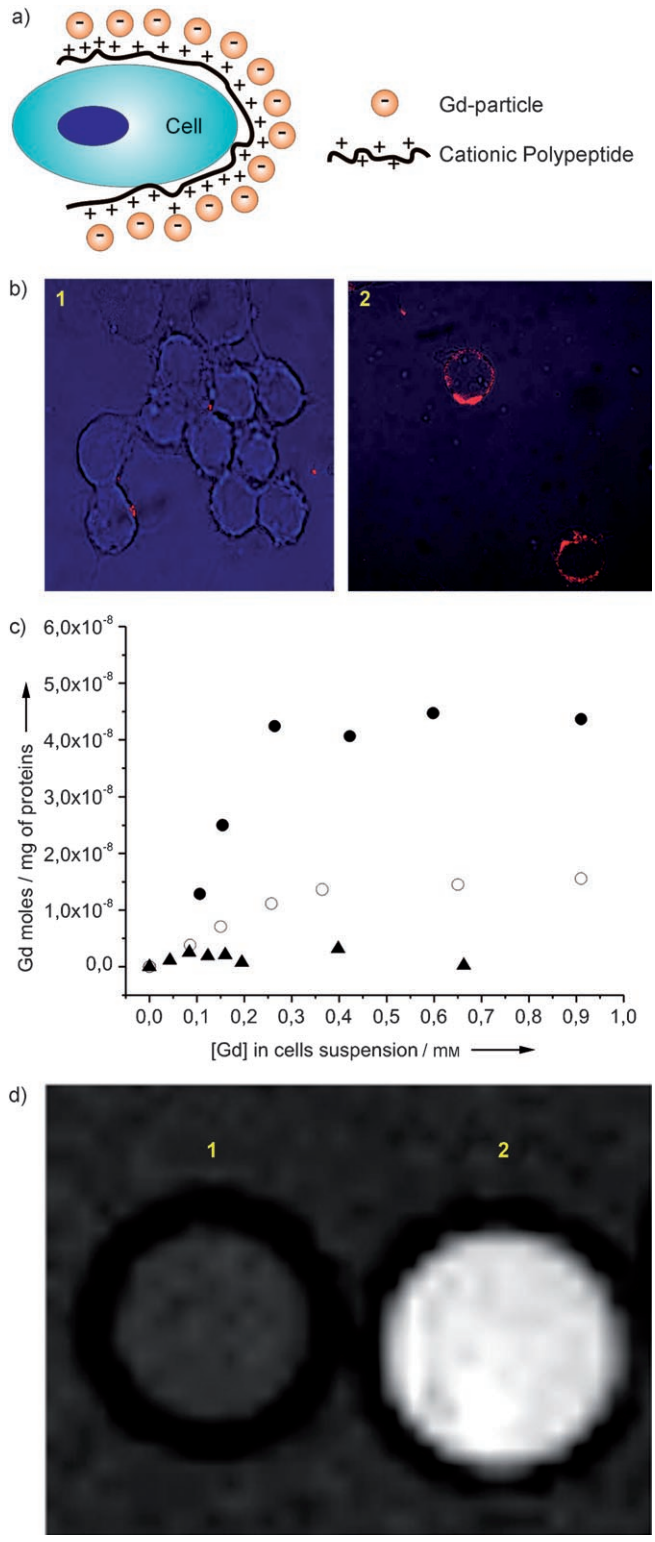

Figure 2. The paramagnetic particles are anchored to the outer cell surface by a cationic polypeptide (polyarginine). a) The negative charges on the cell membrane bind the positively charged polyarginine peptide (MW = 30800) system that also acts as collecting agent for the negatively charged Gd-loaded particles; b) The binding of the supramolecular adduct at the cell membrane through the intermediation of the cationic linker is validated by the confocal microscopy images obtained by using a Gd-containing nanoparticle ([Gd] = 34 μM) doped with a rhodamine-phospholipid and polyarginine (b 2); for comparison the confocal image of cells treated with the same amount of Gd-particles without polyarginine is reported (b 1); c) amount of Gd mg protein determined on the cell surface from cell pellets recovered after short contact (1 min) at low temperature (4 °C) with the cationic polyarginine (30 μg mL^{-1}) and the Gd-particles (●), only the Gd-particles (○), and polyarginine (30 μg mL^{-1}) and Gd-DTPA (▲). Only in the case of simultaneous presence of highly negatively charged particles and the polyarginine, high amounts of Gd accumulated at the cell surface; d) MRI of cellular pellets (approximately 5–6 × 10^6 Neuro-2a cells) in agar. The cells labeled with polyarginine/Gd-nanoparticles (d 2) contains approximately 6 × 10^9 Gd per cell and display a T_1 value of 334 ms to be compared with a T_1 of 2064 ms of the unlabeled cells (d 1).

very high tendency to aggregate in aqueous media. At monomer concentrations as low as 1 × 10^{-5} M the system appears to be present in an aggregated form characterized by a relaxivity (r_{1p}) of 25 mM^{-1} s^{-1} at 298 K and 20 MHz. Upon acquiring r_{1p} values over an extended frequency range (0.01–70 MHz) the resulting NMRD profile (Figure 1 b) shows the typical shape of slowly moving aggregates and it has been fitted assuming the occurrence of local and global motions as previously suggested for related self-assembled Gd-containing systems.[9–11] Light Scattering measurements yield, for these particles, a diameter of approximately 80 nm (with a very low polydispersity), and an aggregation number of approximately 850 (from the determination of apparent molecular weight by light scattering measurements). Thus the high lipophilicity of the substituents in the Gd–AAZTA complex yields very stable particles much larger than the ordinary micelles[11,12] (likely discoid aggregates) that display on their surface a high density of negative charges. As such they do not interact with the cell surface, the latter being characterized by an overall negative charge, though single anphiphilic components may insert into the cellular membrane (Figure 2 b).

One may envisage several ways to anchor a paramagnetic particle on the surface of a cell. Herein, we have exploited a cationic linker that binds to the negative charges either on the particles and the membrane surface (Figure 2 a). Different polycationic species have been successfully tested, namely protamine, polylysine, polyornithine, and polyarginine. Overall, the polyamino acids worked better than protamine and a polyarginine with a polymerization degree of 159 has been selected for further investigations. By measuring the changes in zeta potential (Supporting Information Figure 1) of a cell suspension (approximately 50.000 NEURO-2a cells mL^{-1}) it has been found that the charge saturation point is reached when 7–8 × 10^8 polyarginine molecules per cell have been added. To maintain a good level of cell functionality, the cell labeling experiments have been carried out by using doses of polyarginine corresponding to 15 % of the saturation value.

In these conditions a viability test, using trypan-blue as a cell marker, has been performed resulting in excellent viability (98 %) both for cells treated with polyarginine alone and with polyarginine and Gd-particles at different concentrations.

Cell labeling was performed at 4 °C by addition of different aliquots of Gd-particles to approximately 4–5 × 10^6 NEURO-2a cells suspended in 800 μL of phosphate buffer saline with or without polyarginine (30 μg mL^{-1}). In the presence of polyargi-

nine as a linker, the loading of Gd^{III} is four times higher than that measured for the Gd-micelles alone. Furthermore it has been found that using simple Gd–DTPA instead of the supramolecular Gd-containing micellar system, the Gd^{III} loading is almost zero (Figure 2c).

As shown in the fluorescence confocal image obtained on cells labeled with rhodamine-containing (0.1%) Gd-micelles (34 μM) and polyarginine (2 $\mu g\,mL^{-1}$) (Figure 2b2), the imaging reporter system is bound on the cellular membrane without any evidence of internalization. In Figure 2b1 the confocal image of cells treated with the same amount of rhodamine labeled Gd-micelles without polyarginine is shown. As no fluorescence is visible it is clear that the polycationic linker is necessary for successful cellular labeling and that the imaging reporters are linked outside the cell membrane and not incorporated in the lipid bilayer as monomers. By using 1.1×10^8 polyarginine molecules per cell, it has been found that each cell can load up to approximately 5×10^6 particles. This corresponds to approximately 5×10^9 Gd/cell, well above the threshold for MRI visualization. Furthermore, as the Gd-containing units are anchored on the external surface of cells and not entrapped in endosomes inside the cells, the relaxivity is not quenched increasing the Gd-loading[13] but increases linearly with the quantity of Gd-particles added (Supporting Information Figure 2). Finally, a T_1 weighted MR-image was recorded on a Bruker spectrometer operating at 7.1T on pellets of cells treated with Gd-micelles ([Gd] = 0.3 mM) and polyarginine (30 $\mu g\,mL^{-1}$) (Figure 2d). The labeled cells appear markedly hyperintense with respect to the control cells. From the NMRD data (Figure 1b) it is expected that the contrast enhancement effect would have been much higher at magnetic fields close to 1T.

In summary, the method proposed herein appears to provide a number of advantages with respect to the currently used labeling procedures. For instance the linker may be designed to be activated by a specific component (pH, enzyme) characteristic of the target region. In this way it should be possible to set-up a controlled release of the imaging payload (and eventually of an associated therapeutic agent) at the target. Using this route cells may act as targeting vectors for a variety of imaging/therapeutic payloads including micelles, liposomes, and other nanosized carriers.

Experimental Section

Relaxometric measurements were carried out on a Stelar Spinmaster Relaxometer operating at variable frequencies between 20 and 80 MHz and on a Stelar Field Cycling Relaxometer in the frequency range 0.01–20 MHz. The size and molecular weight of the micellar systems were measured by dynamic light scattering on a Malvern Zetasizer Nano ZS instrument. Cell labeling was carried out on a murine neuroblastoma (NEURO-2a) cell line. Cells were grown in 75 cm^2 flasks in DMEM medium supplemented with FBS (5%), penicillin (100 $U\,mL^{-1}$), and streptomycin (100 $\mu g\,mL^{-1}$). The cells were then seeded in culture dishes (10 cm diameter) at a density of about 1.4×10^6 cells.

After 12 h the medium was removed; the cells (approximately $3–4\times10^6$) were washed three times with ice-cold PBS, and suspended in 800 μL PBS containing variable quantities of Gd-particles. After 5 min at 4 °C cells were washed three times with 1 mL ice-cold PBS and collected in 200 μL PBS, then sonicated and treated with 200 μL of HCl 37%. Upon heating at 120 °C for 16 h in harsh acidic conditions, all Gd^{III} is dissolved as free aquo-ion. By measuring the proton relaxation rate (R_{1obs}) at 20 MHz, 25 °C of these solutions it is possible to determine the Gd^{III} concentration.

MR-imaging was performed on a Bruker300 spectrometer equipped with a microimaging probe operating at 7.1T.

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