Effect of the Intracellular Localization of a Gd-Based Imaging Probe on the Relaxation Enhancement of Water Protons

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Gd-HPDO3A has been internalized into rat hepatocarcinoma cells in the cytoplasm (by electroporation) or in intracellular vesicles (by pinocytosis), respectively. In the former case, the observed relaxation rates are likely dependent upon the amount of internalized paramagnetic complex, whereas in the latter case the relaxation enhancement is “quenched” to a plateau value (about 3 s⁻¹) when the entrapped amount of Gd-chelate is higher than 1 × 10¹⁰ Gd/cell. The observed behavior has been accounted in terms of a theoretical treatment based on equations formally derived by Labadie et al. (J Magn Reson B 1994; 105:99–102). On this basis, entrapment into intracellular vesicles has been treated as a three-site water exchange (extracellular/cytoplasm/vesicle compartments), whereas the cell pellets containing the paramagnetic agent spread out in the cytoplasm can be analyzed by a two-site exchange system. Mag. Reson. Med. 55:491–497, 2006. © 2006 Wiley-Liss, Inc.

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MRI visualization of cells labeled with Gd-based agents appears a promising route for pursuing novel applications in the field of cellular and molecular imaging (1–4). In fact, it has been shown that the problems associated with the intrinsic low sensitivity of the MRI modality can be overcome by the intracellular accumulation of a high number of paramagnetic Gd(III)-chelates. A straightforward method for pursuing the entrapment of 10⁶–10⁹ Gd-chelates per cells consists of incubating, for several hours, the cells in a culture medium containing increasing amounts (from 5 to 100 mM) of Gd-HPDO3A. After this treatment, the cells were washed three times and yield hyperintense spots in MR images. However, it has been noted that the observed relaxivity is “quenched” in the presence of relatively high amounts of internalized Gd-chelates (5–8). This drawback clearly represents a limitation to the proposed method of cell labeling and must be investigated in more detail. In order to ascertain whether the observed relaxivity quenching is dependent upon the localization of the paramagnetic agent inside the subcellular vesicles, it has been deemed of interest to carry out parallel internalization experiments using the electroporation procedure. The latter technique causes the formation of transient hydrophilic pores on the cell membrane upon the application of suitable electric pulses between two electrodes placed into the cell suspension (9). Electroporation is widely used in cellular biology laboratories as a route to deliver hydrophilic xenobiotics into the cellular cytoplasm (10,11). In this paper, the relaxation enhancement induced by Gd-HPDO3A internalized in HTC (rat hepatocarcinoma) cells into subcellular vesicles (by pinocytosis) or into the cellular cytoplasm (by electroporation) have been compared. The obtained results allow us to forward an explanation on why the entrapment into vesicles leads to a quenching of the attainable relaxation enhancements.

MATERIALS AND METHODS

Gd-HPDO3A (Prohance) was kindly provided by Bracco S.p.A. (Milan, Italy). Eu-HPDO3A was synthesized as previously described (5).

Dulbecco’s modified Eagle’s medium (DMEM) medium and penicillin/streptomycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum was purchased from Cambrex (Rutherford, NJ, USA).

HTC cells were obtained by Istituto Nazionale per la Ricerca sul Cancro (Genova, Italy).

Cell Labeling

The cells (rat hepatocarcinoma cell line (HTC)) were grown in 75-cm² flasks in a humidified CO₂ incubator at 37°C, under air/CO₂ 95/5 atmosphere in DMEM-F12 medium supplemented with 5% fetal bovine serum, 100 U/1mL penicillin, and 100 mg/mL streptomycin. The cells were then seeded in 75-cm² flasks at a density of about 25000 cells/cm². After 24 h cells were washed and subjected to the uptake experiments.

Pinocytosis

In each experiment, about 5–6 million cells were incubated at 37°C for 16 h in culture medium containing increasing amounts (from 5 to 100 mM) of Gd-HPDO3A. After this treatment, the cells were washed three times...
with 10 mL ice-cold phosphate-buffered saline (PBS), detached with trypsin/EDTA, and transferred into a 5-mm NMR tube.

**Electroporation**

In each experiment, about 5–6 million cells were detached from the cell culture flask with a trypsin/EDTA solution and placed in an electroporation cuvette containing increasing concentrations of Gd-HPDO3A (from 2 to 100 mM) in 0.8 mL of PBS. The electroporation was performed using Gene Pulser II electroporation system (Bio-Rad Laboratories, Hercules, CA, USA), applying a single shock at 0.2 kV, with 950 microF capacitance and with a time constant of about 10 ms. After 30 h, cells were washed three times with 10 mL ice-cold PBS and transferred into a 5-mm NMR tube.

**Relaxation Rate (1/T₁) Measurements**

The 5-mm NMR tubes containing the cell suspensions were placed in a centrifuge at 1000g for 5 h. The supernatant was carefully removed and the T₁ of the cellular pellets was measured at 0.5 T and 25°C on a Stelar Spinmaster spectrometer (Stelar, Mede, Italy) by means of the inversion-recovery (IR) pulse sequence. The Gd³⁺ concentration in each pellet was determined as described previously (5). The determination of water exchange rates (kᵢ, and kᵢ⁺) across the cell membrane have been obtained by measuring water proton T₁ of cell pellets containing GdHPDO3A in the extracellular compartment. To this purpose, in each experiment, ca. 5–6 × 10⁶ cells have been suspended in ice-cold PBS (4°C) added with increasing amounts of Gd-HPDO3A (from 20 to 60 mM). The cell pellets have been obtained by centrifugation (at 4°C). The relaxation data have been analyzed in terms of the 2SX model (12) using the longitudinal relaxation measurements of unlabeled pellets at different cytosolic, compartments for the HTC pellets have been determined as described previously (5). The determination of water exchange rates (kᵢ, and kᵢ⁺) across the cell membrane have been obtained by measuring water proton T₁ of cell pellets containing GdHPDO3A in the extracellular compartment. To this purpose, in each experiment, ca. 5–6 × 10⁶ cells have been suspended in ice-cold PBS (4°C) added with increasing amounts of Gd-HPDO3A (from 20 to 60 mM). The cell pellets have been obtained by centrifugation (at 4°C). The relaxation data have been analyzed in terms of the 2SX model (12) using the relaxation rate values of the cytosolic and the extracellular compartments of unlabeled cells (0.8 and 0.5 s⁻¹, respectively) and the relaxivity of GdHPDO3A (4.2 s⁻¹ mM⁻¹) as fixed parameters.

**Determination of R¹_{cy} and R¹_{ex}**

The relaxation rate of the extracellular, R₀_{ex} and the cytosolic, R₀_{cy} compartments for the HTC pellets have been determined by measuring the longitudinal relaxation rate of water protons of unlabeled pellets at different cytosolic volume fraction, x_{cy} / (1− x_{cy}).

The x_{cy} / (1− x_{cy}) values have been determined by multiplying the cell number contained in a known volume of each pellet times a cell volume of 1.5 × 10⁻¹² L. The cell volume has been calculated on the basis of a mean cell diameter of 14 μm as evaluated in the confocal microscopy images.

The time evolution of the magnetization in the IR experiment on unlabeled HTC pellets is monoexponential (fast-exchange limit) and the relaxation rate values for the two compartments have been obtained from the equation

\[
R₁ = R₀_{cy} x_{cy} + R₀_{ex} (1 - x_{cy}). \tag{1}
\]

**RESULTS AND DISCUSSION**

Gd-HPDO3A is a highly hydrophilic and well-tolerated agent, currently used in clinical practice (Prohance, Bracco Imaging S.p.A). Internalization by pinocytosis has been carried out by incubating HTC cells (5–6 million) for 16 h at 37°C in the presence of increasing amounts of Gd-HPDO3A (0–100 mM). As Gd-HPDO3A does not show any binding interaction to cell membranes, its cellular uptake occurs through pinocytosis (5). To assess the intracellular distribution of the contrast agent, 5 × 10⁵ HTC cells were incubated in the presence of a 50 mM solution of Eu-HPDO3A. In fact, it has been previously shown (13–14) that Eu-HPDO3A has good fluorescent properties and can be visualized by the microscopic techniques currently used in cellular biology. The images obtained with confocal microscope (Fig. 1) clearly show that Eu-HPDO3A is confined into endosomic vesicles in the perinuclear region. This result is consistent with previous results obtained on endothelial progenitor cells (5). Several examples of subcellular compartmentalization have been reported when the magnetic label is represented by iron-oxide systems (15–18). Moreover, confocal images obtained from neural stem cells labeled with a bimodal contrast agent, consisting of Gd-DTPA and fluorescent rhodamine moieties, show analogous compartmentalization (3).

In parallel to the endosomic entrapment, the uptake of Gd-HPDO3A into the cellular cytoplasm has been pursued by electroporation. These experiments have been carried out applying an electric pulse of 0.2 kV (12 ms long) to a cell suspension containing the imaging probe in the same concentration range used for pinocytosis (0–100 mM). This treatment leads to the formation of pores on the cell membrane in the frame of less than a second, whereas their rescaling takes minutes. Through these pores small molecules as Gd-HPDO3A can diffuse directly into the cytoplasm (19–21).

Figure 2 reports the amount of internalized Gd³⁺ (expressed in nanomoles per mg of total protein) versus the millimolar concentration of Gd-HPDO3A present in the medium. No saturation effect is detected using both inter-
nalization procedures and the amount of the uptaken Gd$^{3+}$/H$_{11001}$ is linearly proportional to the concentration of the imaging probe in the medium. Furthermore, the data reported in Fig. 2 indicate that, for the same concentration of Gd-HPDO3A in the medium, the amount of internalized complex after 16 h of incubation is the same as that found after a single electroporating shock.

An important issue is the biologic effect associated with the entrapment of Gd-HPDO3A into hepatoma cells. The percentage viability of HTC incubated with Gd-HPDO3A at 37°C for 16 h (pinocytic uptake), evaluated by the trypan blue based assay, was greater than 96% for all tested concentrations. Conversely, the application of an electric pulse on the cell suspension is a rather harsh treatment and ca. 30% of the cells were destroyed during the electroporation procedure. The resulting debris is easily separated by centrifugation. The remaining cells, when subjected to the trypan blue test, showed a viability of 70–75%.

The relaxometric properties of the Gd-labeled cellular pellets have been investigated through the measurement of their longitudinal proton relaxation rate at 0.47 T and 25°C (Fig. 3).

The relaxation rate of the cells labeled by pinocytosis shows a saturation effect upon increasing of the amount of internalized probe (Fig. 3, circles) with a $R_1$ limiting value of about 3 s$^{-1}$. The time evolution of the longitudinal magnetization ($M_z$) in the inversion recovery (IR) experiment for the cellular pellets labeled by pinocytosis was monoexponential in the whole range of concentrations of the internalized probe. The relaxometric behavior shown by the cellular pellets labeled by electroporation was markedly different. In fact, the recovery of $M_z$ was monoexponential only when the amount of internalized probe was lower than $2 \times 10^9$ Gd$^{3+}$/cell and biexponential for higher concentrations. Consequently, we analyzed the latter IR profiles using a simple biexponential model and Fig. 3 reports the two $R_1$ values obtained for each pellet. It is likely that the two $R_1$ values reflect the relaxometric properties of the two main compartments, intra- and extracellular, of the cellular pellet (see below). Furthermore, it is worth noting that the $R_1$ values for electroporated cells are markedly higher than the corresponding values for the pellets labeled by pinocytosis and, even more important, they are linearly dependent upon the amount of the internalized complex (Fig. 3, open and filled squares).

The different relaxation enhancement efficacy displayed by the HTC pellets, as a function of the internalization route, was confirmed by acquiring MR images of phantoms containing the labeled cells dispersed in agar. For the same amount of internalized agent, the cells labeled by electroporation appear invariably hyperintense than those ones labeled by pinocytosis (Fig. 4a). These experiments allow us to establish that the minimum number of HTC cells labeled with GdHPDO3A (ca. $4 \times 10^{10}$ molecules/cell) detectable by MRI is ca. 500 cells/µL in the case of electroporation and ca. 5000 cells/µL for pinocytosis (Fig. 4b).

Having established that the relaxation enhancement efficacy of Gd-HPDO3A relies on the type of intracellular localization of the imaging probe, a quantitative treatment of the relaxation data has been undertaken in order to validate a proper model for the observed behavior.

A cellular pellet can be considered a multisite system where the water molecules are distributed in the extra- and intracellular (mainly cytosolic) compartments. Such compartments are separated by the cellular membrane, whose water permeability is crucial for determining the relaxometric behavior of the whole pellet. The effect of the water exchange across the membrane on the relaxation rate of water protons in a two-site system can be quantitatively described by a theoretical model, reported as the 2SX model, developed by Labadie et al. for analyzing $R_1$ data of tissues in the presence of an extracellular contrast agent (12).

![FIG. 1. Confocal optical section of the cells showing that the fluorescent Eu-HPDO3A accumulates in the subcellular vesicles around nuclei.](image)

![FIG. 2. The internalization of Gd-HPDO3A into HTC cells. About 5–6 million cells were (●) incubated at 37°C for 16 h (pinocytosis) or (□) electroporated applying a pulse of 0.2 kV. Both electroporation and pinocytosis were performed in the presence of increasing concentrations of contrast agent (Gd-HPDO3A).](image)
FIG. 3. Water protons longitudinal relaxation rates at 20 MHz and 25°C of cell pellets labeled with Gd-HPDO3A by pinocytosis (circles) or by electroporation (squares and upper triangles). For electroporated cells, the $M_z$ recovery was monoexponential only for cell containing less than $2 \times 10^9$ Gd/cell (single $R_1$, upper triangles) and biexponential at higher amounts of internalized probe (two $R_1$ values, open and filled squares). The solid curves represent the best data fitting to the hyperbolic (pinocytosis) and linear (electroporation) behavior.

According to this model, the time evolution of $M_z$ in a two-site system is dependent on the relationship between the absolute values of a “relaxation” term, $R_{1\text{-cy}} - R_{1\text{-ex}}$, also defined as NMR “shutter-speed,” and an “exchange” term, $k^{\text{cy/ex}} + k^{\text{ex/cy}}$, where $R_{1\text{-cy}}$ and $R_{1\text{-ex}}$ represent the relaxation rates in the two compartments in the absence of exchange and $k^{\text{cy/ex}}$ and $k^{\text{ex/cy}}$ refer to the “intra $\rightarrow$ extra” and to the “extra $\rightarrow$ intra” water exchange rates, respectively. Such exchange rates are correlated, accordingly to the mass balance, through the volume fraction values ($\chi_v$) of the two compartments:

$$\chi^{\text{cy/ex}}_v k^{\text{cy/ex}} = \chi^{\text{ex/cy}}_v k^{\text{ex/cy}}. \quad [2]$$

On the basis of this model, a monoexponential time-course of $M_z$ is expected when a fast-exchange regime occurs, i.e., when the condition $R_{1\text{-cy}} - R_{1\text{-ex}} \ll k^{\text{cy/ex}} + k^{\text{ex/cy}}$ is met. In this case the analysis of the IR data provides a single $R_1$ value that corresponds to the average between $R_{1\text{-cy}}$ and $R_{1\text{-ex}}$ weighted by the volume fractions ($\chi^{\text{cy/ex}}_v$ and $\chi^{\text{ex/cy}}_v$) of the two sites. Conversely, when there is no exchange between the two compartments, the recovery of $M_z$ will be biexponential (provided that $\chi^{\text{cy/ex}}_v$ and $\chi^{\text{ex/cy}}_v$ values are comparable), thus allowing an accurate determination of both $R_{1\text{-cy}}$ and $R_{1\text{-ex}}$ values (and also $\chi^{\text{cy/ex}}_v$ and $\chi^{\text{ex/cy}}_v$) through a simple biexponential analysis of the IR data.

In between, there is the intermediate-exchange region in which the time evolution of $M_z$ can be still biexponential, but the relaxation rates obtained from the fitting of the IR data can be “contaminated” by the exchange occurring between the two compartments, thus resulting in apparent $R_1$ values that are larger than the real relaxation rates (12).

It is noteworthy that the exchange term is mainly dependent upon the biologic properties of the cellular pellet (cell type and relative volume fractions of the two compartments), whereas the relaxation term is markedly affected by the presence of the paramagnetic probe. Therefore, when the imaging probe is distributed into cytosol the $R_{1\text{-cy}} - R_{1\text{-ex}}$ term will increase linearly as a function of the amount of the internalized agent, thus leading, for a sufficiently high membrane permeability, to a shift from a fast-exchange regime to an intermediate-exchange regime. This is what we observed for the cells labeled by electroporation: when the amount of internalized Gd-HPDO3A is smaller than $2 \times 10^9$ molecule/cell the time evolution of $M_z$ is monoexponential, whereas for higher amounts the magnetization recovery behavior becomes biexponential.

In order to perform a quantitative analysis, the assessment of the membrane permeability of HTC pellets is crucial. For this reason, a series of $R_1$ measurements (0.47 T and 25°C) have been acquired by adding increasing amounts (20–60 mM) of Gd-HPDO3A to the extracellular space of a HTC cellular pellet. In order to avoid the inter-

FIG. 4. (a) $T_1$-weighted spin echo image (TR/TE/NEX = 100/3.2/24, FOV 3.1 cm, 1 slice 1 mm) of an agar phantom containing HTC cells labeled with GdHPDO3A internalized by pinocytosis (P) or electroporation (E). Cells are dispersed in agar at different densities: (1) 1 x $10^4$ cells/μL, (2) 5 x $10^3$cells/μL, (3) 1 x $10^5$cells/μL. (b) $T_1$-weighted spin echo image (TR/TE/NEX = 500/3.2/4, FOV 3.3 cm, 1 slice 1 mm) of an agar phantom containing HTC cells (5 x $10^5$cells/μL) labeled with GdHPDO3A internalized by pinocytosis or electroporation.
nalization of the metal complex, the addition was performed at 25°C and the relaxometric investigation was carried out within 30 min. Then, the IR data were analyzed according to the 2SX model using, as fitting constraints, the relaxation rate of the intra- and the extracellular compartments in the absence of the paramagnetic probe (0.8 and 0.5 s⁻¹, respectively, see Experimental), and the relaxivity of Gd-HPDO3A (4.2 s⁻¹ mM⁻¹). The analysis yielded an average $k^\text{cy/ex}$ value of 14 ± 6 s⁻¹ with an average cytosolic volume fraction, $\chi^\text{cy/ex}$, of 0.32 ± 0.05.

The obtained $k^\text{cy/ex}$ value is, as expected, smaller than the corresponding value observed for a suspension of human erythrocytes (50–100 s⁻¹) (22.23) whose membrane is known to have the highest water permeability.

As the electroporation route localizes the imaging probe in the cellular cytosol, the 2SX model has been also used for analyzing the IR data obtained for the electroporated cells. Interestingly, by using as fitting constraint the obtained $k^\text{cy/ex}$ value (besides the other parameters mentioned above), a slightly larger relaxivity value (5.2 s⁻¹ mM⁻¹) for the internalized complex has been found. This increase appears reasonable as it likely reflects the higher viscosity of the cytosol with respect to the extracellular compartment. As far as the $\chi^\text{cy/ex}$ value is concerned, the results of the fitting procedure yielded an average value of 0.39 ± 0.1 in close agreement with the value previously obtained for the same pellet type in the $k^\text{cy/ex}$ determination experiment.

In the case of pinocytosis, the imaging probe is confined into a third whose volume fraction is so small (<1%) (15) to be not directly detectable in the IR experiment. However, it is straightforward that the relaxation rate of the water protons in the cytosol will be affected by the Gd-labeled vesicle to an extent that is dependent on the water permeability across the vesicle, the number of vesicles per cell, and the concentration of Gd-HPDO3A inside the vesicles. On this basis, the quantitative analysis of the IR data for the cellular pellets labeled by pinocytosis has been carried out using a modified version of the 2SX model, which may be called pseudo-3SX, in which the $R_1$ value of the intracellular compartment is controlled by the water permeability of the vesicle membrane.

According to this model, the relaxation rate of the cytosolic compartment, $R_{1-cy}$, in the presence of Gd-labeled vesicles, can be calculated from the equation

$$R_{1-cy} = \frac{1}{2}[R_{0-cy} + r_1^\text{cy}[\text{GdL}]_\text{cy} + R_{1-cy}^0 + k^\text{cy/w} + k^\text{ve/cy}]$$

$$- \frac{1}{2}[[R_{0-cy} - r_1^\text{cy}[\text{GdL}]_\text{cy} - R_{1-cy}^0 + k^\text{cy/w} - k^\text{ve/cy}]^2 + 4k^\text{cy/w}\frac{k^\text{ve/cy}}{1-k^\text{ve/cy}}^{12}, \quad [3]$$

where $R_{0-cy}$ and $R_{0-ve}$ are the relaxation rate of the vesicle and the cytosolic compartments in the absence of the paramagnetic probe, respectively, $r_1^\text{cy}$ is the millimolar relaxivity of the imaging probe inside the intracellular vesicle, $[\text{GdL}]_\text{cy}$ is the millimolar intraendosomal concentration of the imaging probe, and $k^\text{ve/cy}$ and $k^\text{cy/w}$ refer to the “vesicle → cytosol” and to the “cytosol → vesicle” water exchange rates, respectively. These exchange rates are correlated through the equilibrium mass balance (see Eq. [1]) and, therefore, $k^\text{ve/cy}$ can be easily expressed as a function of $k^\text{cy/w}$ and $\chi^\text{cy/w}$ (that indicates the volume fraction of the cytosol over the whole cellular volume):

$$\chi^\text{cy/w}k^\text{cy/w} = \chi^\text{ve/cy}k^\text{ve/cy} \xrightarrow{k^\text{cy/w}} \frac{k^\text{cy/w}k^\text{ve/w}}{(1 - \chi^\text{cy/ex})}. \quad [4]$$

The intravesicle concentration of the imaging probe can be expressed in terms of its total concentration in the whole cellular pellet:

$$[\text{GdL}]_\text{ve} = \frac{[\text{GdL}]_\text{cy}}{\chi^\text{cy/w}A^\text{cy}/V^\text{cy}} \xrightarrow{k^\text{cy/w}} k^\text{ve/w} = P^\text{w}A^\text{w}/V^\text{w}, \quad [5]$$

where $P$ is the water permeability coefficient of the membrane (cytolemma, cy, or vesicle, ve), $A$ is the surface area of the corresponding membrane, and $V^\text{cy}$ is the volume of the cytosolic compartment. As the vesicles originate from the “blebbing” of cytolemma, the permeability of their membrane can be reasonably assumed to correspond to that one of the cytolemma ($P^\text{w} = P^\text{cy}$). Therefore, $k^\text{cy/w}$ is related to the value of $k^\text{cy/ex}$ through a factor that depends on the ratio between the surface area of the two membranes,

$$k^\text{cy/w} = k^\text{cy/ex} \frac{A^\text{ve}}{A^\text{cy}} = k^\text{cy/ex} A^\text{ve}\frac{\sigma^\text{w}}{A^\text{cy}}, \quad [6]$$

where $\sigma^\text{w} \equiv$ is the superficial area of the membrane of a single vesicle and $n^\text{ve}$ is the number of vesicles per cell.

Finally, by applying the usual 2SX model, we use the value of $R_{1-cy}$ obtained from Eq. [3] to calculate the relaxation rates of the intracellular ($R_{1-cy}$) and of the extracellular ($R_{1-ve}$) compartments on the cellular pellets labeled by pinocytosis,

$$R_{1-cy} = \frac{1}{2}[R_{1-ve} + R_{1-cy} + k^\text{ex/cy} + k^\text{ex/ve}]$$

$$+ \frac{1}{2}[[R_{0-ve} - R_{1-ve} - k^\text{ex/cy} - k^\text{ex/ve}]^2 + 4k^\text{ex/cy}k^\text{ex/ve}]^{12}, \quad [7]$$

where $R_{1-ve}$ and $R_{1-cy}$ are the relaxation rate of the vesicle and the paramagnetic probe, respectively, $r_1^\text{cy}$ is the millimolar relaxivity of the imaging probe inside the intracellular vesicle, $[\text{GdL}]_\text{cy}$ is the millimolar intraendosomal concentration of the imaging probe, and $k^\text{ve/cy}$ and $k^\text{cy/w}$ refer to the “vesicle → cytosol” and to the “cytosol → vesicle” water exchange rates, respectively. These exchange rates are correlated through the equilibrium mass balance (see Eq. [1]) and, therefore, $k^\text{ve/cy}$ can be easily expressed as a function of $k^\text{cy/w}$ and $\chi^\text{cy/w}$ (that indicates the volume fraction of the cytosol over the whole cellular volume):

$$\chi^\text{cy/w}k^\text{cy/w} = \chi^\text{ve/cy}k^\text{ve/cy} \xrightarrow{k^\text{cy/w}} \frac{k^\text{cy/w}k^\text{ve/w}}{(1 - \chi^\text{cy/ex})}. \quad [4]$$

The intravesicle concentration of the imaging probe can be expressed in terms of its total concentration in the whole cellular pellet:

$$[\text{GdL}]_\text{ve} = \frac{[\text{GdL}]_\text{cy}}{\chi^\text{cy/w}A^\text{cy}/V^\text{cy}} \xrightarrow{k^\text{cy/w}} k^\text{ve/w} = P^\text{w}A^\text{w}/V^\text{w}, \quad [5]$$

where $P$ is the water permeability coefficient of the membrane (cytolemma, cy, or vesicle, ve), $A$ is the surface area of the corresponding membrane, and $V^\text{cy}$ is the volume of the cytosolic compartment. As the vesicles originate from the “blebbing” of cytolemma, the permeability of their membrane can be reasonably assumed to correspond to that one of the cytolemma ($P^\text{w} = P^\text{cy}$). Therefore, $k^\text{cy/w}$ is related to the value of $k^\text{cy/ex}$ through a factor that depends on the ratio between the surface area of the two membranes,

$$k^\text{cy/w} = k^\text{cy/ex} A^\text{ve}/A^\text{cy} = k^\text{cy/ex} A^\text{ve}\frac{\sigma^\text{w}}{A^\text{cy}}, \quad [6]$$

where $\sigma^\text{w} \equiv$ is the superficial area of the membrane of a single vesicle and $n^\text{ve}$ is the number of vesicles per cell.

Finally, by applying the usual 2SX model, we use the value of $R_{1-cy}$ obtained from Eq. [3] to calculate the relaxation rates of the intracellular ($R_{1-cy}$) and of the extracellular ($R_{1-ve}$) compartments on the cellular pellets labeled by pinocytosis,
and the time evolution of $M_Z$ in the IR experiment,

$$M_{a0} = M_0 \{1 - 2[(1 - a_m)e^{(-t_R - a_m)} + a_m e^{(-t_R - a_m)}]\}. \quad [11]$$

In this set of equations, $R_{1-ex}$ is the relaxation rate of the extracellular site for the unlabeled pellet (0.5 s$^{-1}$), $M_n$ is the Boltzmann equilibrium value of the magnetization for the whole pellet, $M_Z$ is the magnetization value at $t$ time, whereas the other parameters have been already defined previously. Also in this case the two exchange rates are related through the corresponding volume fraction values of the two compartments:

$$\chi_{cy/ex} = \frac{\chi_{ex}}{\chi_{cy}} \frac{k_{cy/ex}}{k_{ex/cy}} \rightarrow k_{ex/cy} = \frac{k_{cy/ex} \chi_{ex}}{(1 - \chi_{cy})}, \quad [12]$$

On this basis, the monoexponential IR data for the cellular pellets labeled by pinocytosis have been analyzed accordingly to the pseudo-3Sx model by fixing the relaxation rate values for the extracellular compartment (0.5 s$^{-1}$), for the intravesicle (in the absence of the imaging probe) and cytosol compartments (0.8 s$^{-1}$), and by using a $\chi_{cy/ex}$ value of 0.99, an intravesicle relaxivity for Gd-HPDO3A ($x_1^{ex}$) of 5.2 s$^{-1}$ mM$^{-1}$, and a $k_{cy/ex}$ value of 14 s$^{-1}$ as obtained from the previous analysis.

The resulting average values for $k_{cy/ex}$ and for the intracellular volume fraction, $\chi_{cy/ex}$ were of 19.5 ± 7.6 s$^{-1}$ and 0.4 ± 0.05, respectively.

On the basis of this result, a $k_{cy/ex}/k_{ex/cy}$ ratio of ca. 1.4 can be calculated. This number corresponds to the $A^n/A^y$ ratio (see Eq. [7]) and it allows an estimation of the size of the vesicles if $A^y$ and $n^y$ values are known. From the diameter of an HTCC cell (ca. 14 μm, see Experimental) it is possible to calculate an $A^y$ value of 615 μm$^2$ that corresponds to a $A^n$ value of ca. 860 μm$^2$. By considering 100 vesicles per cell, (15) then a diameter of about 1.6 μm can be obtained for a single vesicle. This value is within the reported size range for such subcellular organelles (15).

In summary, when the intravesicle concentration of the imaging probe is large (thus resulting in a very high intravesicle relaxation rate), the exchange regime between vesicles and cytosol becomes intermediate/slow and the relaxation rate of the cytosol compartment is only partly enhanced by the presence of the labeled vesicles. In other words, the water exchange across the vesicle membrane sensibly limits the relaxation rate of the cytosolic water protons, if compared with the same amount of probe dissolved in the cytosol. The quenching effect of the exchange on the relaxation rate of cytosolic water protons is then the responsible factor for the saturation of the relaxation rate observed at high concentrations of the internalized probe (Fig. 3, circles) for the HTCC pellets labeled by pinocytosis.

Finally, in such pellets, the relatively low relaxation rate values of the cytosol compartment makes the exchange regime with the extracellular compartments fast and, consequently, a monoexponential recovery of $M_Z$ is observed.

**CONCLUSIONS**

The obtained results remark the importance of the procedure used for labeling cells and demonstrate that the cytosol distribution of the probe yields higher relaxing efficiency, thus allowing the MRI detection of a smaller number of cells with respect to the entrapment into subcellular vesicles. This observation may be particularly useful in the development of a novel and more sensitive MR imaging probe for molecular imaging protocols dealing with in vivo cell targeting or in vitro cell labeling of stem cells or other transplantable cells.

**REFERENCES**


