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# Detection and Quantification of Lanthanide Complexes in Cell Lysates by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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**Gadolinium (III) complexes are under intense scrutiny as contrast agents for magnetic resonance imaging. Although currently used mainly as extracellular agents, there is a growing interest to exploit their contrast enhancing ability in the intracellular environment. To ascertain the preservation of their chemical integrity upon the intracellular entrapment, it is necessary to have a method for their dosage in the cell lysates. Herein, a mass spectrometric method for detection and quantification of gadolinium complexes in cell lysates is reported. The detection by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was carried out by using a non-acidic matrix (2,4,6-trihydroxyacetophenone), which does not allow any leakage of gadolinium from the complex. Quantification has been possible by using as an internal standard an ytterbium complex with the same ligand of the analyte. Ytterbium was chosen because, among the lanthanides, it is the one with the isotopic distribution pattern the most similar to that of gadolinium. Sensitivity was enough to detect low micromolar quantities of a cationic complex and high micromolar quantities of a neutral complex in cell lysates of rat hepatoma cells. In the case of anionic complexes, sensitivity was too low for quantitative analysis. To the best of our knowledge, this is the first report concerning the quantification of metal complexes by MALDI-TOF-MS.**

The advent of magnetic resonance imaging (MRI)[1] for clinical diagnosis has brought an increasing interest in design and synthesis of paramagnetic complexes for their potential use as contrast enhancers.[2] Among the paramagnetic ions, some lanthanides, and particularly gadolinium (III),[3] have been shown to be the most useful for this application because they possess a large magnetic moment and a long electronic relaxation time.[4] Highly stable Gd chelates are currently used in clinical settings as reporters of blood flow and organ perfusion. The next generation will deal with systems endowed with targeting capabilities, and some applications will involve imaging probes that are able to be specifically entrapped into cells.

Recently, Gd complexes have also been proposed for neutron capture therapy (NCT).[5−7] In the latter case, entrapment into the cell is mandatory for the success of the therapy because neutron-activated Gd nuclei emit Auger electrons whose cell damaging effects are confined to few micrometers.

Both for therapeutic and diagnostic applications, the determination of the amount of internalized intact Gd complex is of fundamental importance for monitoring the efficacy of the proposed method. Actually, the determination of Gd ions would have been much easier, but it may be misleading because the biological environment may lead to partial metal decomplexation. Therefore, one has to discard very sensitive techniques, such as inductively coupled plasma mass spectrometry (ICP-MS), X-ray spectromicroscopy, and time-of-flight secondary ion mass spectrometry (TOF-SIMS), because they are not able to discriminate between Gd complexes and other gadolinium-containing compounds arising from the speciation of Gd ions released from the complex.

The specific uptake of Gd complexes has already been shown in several cell cultures.[3,8,9] Among the techniques which can be used for quantification of complexes in cell lysates, nuclear magnetic resonance (NMR) has an inherently low sensitivity; high-pressure liquid chromatography (HPLC) needs further work for optimization of separation procedures; and electrospray ionization mass spectrometry (ESI-MS) has a low tolerance toward sample contaminants, especially salts.

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MALDI-TOF-MS, instead, is a simple, rapid, and highly specific technique that requires minimal sample volumes and no sample pretreatment and has a high sensitivity and a better tolerance toward salts than ESI-MS.

Quantitative analysis in MALDI-TOF-MS experiments has been carried out so far by adding to the samples a stable isotope-incorporated internal standard, chemically equivalent to the target molecule,[10−15] to minimize any variability in sample/matrix co-crystallization and desorption/ionization processes. This approach is, however, very expensive, especially when a mass shift of at least 10 Da is needed, as it would be in the case of Gd complexes (whose isotopic pattern is composed of at least six predominant peaks).

When dealing with complexes, an effect analogous to the isotope-labeling, but simpler and cheaper, can be pursued by using, as an internal standard, a complex with the same ligand of the analyte to be quantified that contains a different metal ion. For the quantitative analysis of Gd complexes, we found that ytterbium could be ideal as an internal standard metal because it is the lanthanide ion with an isotopic distribution the most similar to gadolinium, and their masses are different enough (16 Da) to avoid any overlap between standard and analyte peaks.

For an accurate determination of metal complex content in cell lysates, it is also important to choose experimental conditions which avoid the leakage of metal ions from the complexes during both lysis and MALDI experiments. In this context, the use of the appropriate matrix is crucial.

In this work, we report the determination of intracellular amounts of two Gd complexes differing in the residual charge, Gd−DTPA−BAG being positively charged and Gd−HPDO3A neutral under lysis conditions.

## MATERIALS AND METHODS

**Materials.** The DMEM-F12 medium and fetal bovine serum (FBS) were from Cambrex Bioscience (Verviers, Belgium). Glutamine, penicillin/streptomycin, and all other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Gd−HPDO3A (Prohance) is a Bracco Imaging (Milan, Italy) trademark. Both Gd−HPDO3A complex (white powder) and HPDO3A ligand were kindly provided by Bracco Imaging.

**Synthesis of Yb−HPDO3A.** Yb−HPDO3A was synthesized by adding an excess of ytterbium (III) oxide to an aqueous solution of HPDO3A (10-(2-hydroxypropyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid). The reaction mixture was stirred at 70 °C for 8 h. Complex formation was followed by measuring $^1$H NMR spectra up to the complete disappearance of the free ligand signals. The excess of $Yb_2O_3$ was removed by formation of $Yb(OH)_3$ at basic pH, followed by centrifugation and filtration (on a 0.2-$\mu$m syringe filter) of the resulting suspension. The metal complex containing solution was then lyophilized.

**Synthesis of Gd− and Yb−DTPA−BAG.** DTPA (diethylenetriaminepentaacetic acid) was dissolved in pyridine and mixed with an excess of acetic anhydride. The suspension was heated at 65 °C for 3 h, then cooled and filtered. The solid (DTPA-bisanhydride) was washed with acetic anhydride, dichloromethane, and diethyl ether and lyophilized. Agmatine (4-guanidinobutylamine) sulfate was dissolved in water, and agmatine was extracted in 1-butanol at pH 13. The solvent was removed under reduced pressure, and the residue was dissolved in dimethyl sulfoxide (DMSO) at 50 °C. DTPA-bisanhydride was then dissolved in DMSO and added to agmatine at 50 °C. The reaction mixture was stirred at 50 °C for 10 h. An excess of acetone was then added, and the solid DTPA−BAG (*N*,*N*′-(4-guanidinobutyl)diethylenetriaminepentacetic acid bisamide) was then filtered, washed with acetone, dissolved in water at pH 2, and purified onto an Amberlite XAD 1180 resin, by elution with a gradient of methanol from 0 to 100%. Lyophilized DTPA−BAG was then dissolved in water at pH 6.5 and mixed with an excess of gadolinium (III) or ytterbium (III) chloride. The reaction mixture was stirred at 70 °C for 8 h. Complex formation was followed by measuring $^1$H NMR spectra up to the complete disappearance of the free ligand signals. The excess of gadolinium (III) or ytterbium (III) chloride was removed by formation of $Gd(OH)_3$ or $Yb(OH)_3$ at basic pH, followed by centrifugation and filtration (on a 0.2-$\mu$m syringe filter) of the resulting suspension. The metal complex containing solution was then lyophilized.

**Cell Culture.** HTC (rat hepatoma cells) were grown in 75-cm$^2$ flasks in a humidified incubator at 37 °C and at $CO_2$/air (5:95 v/v) in DMEM-F12 medium supplemented with 5% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 $\mu$g/mL streptomycin. Cells were seeded in 10-cm Petri dishes at a density of 15 000 cells/cm$^2$. After 24 h, cells were ready for the uptake experiments.

**Uptake Experiments.** HTC cells seeded in 10-cm Petri dishes were used to carry out the uptake experiments with either Gd−DTPA−BAG or Gd−HPDO3A. After removal of the culture medium, the cells were washed with 5 mL of phosphate buffered saline (PBS) and then 5 mL of Earl's balanced salt solution (EBSS: $CaCl_2$ 0.266 g/L, KCl 0.4 g/L, NaCl 6.8 g/L, glucose 1 g/L, $MgSO_4$ 0.204 g/L, $NaH_2PO_4$ 0.144 g/L, and $NaHCO_3$ 1.1 g/L pH 7.4) were added. HTC cells were incubated for 6 h at 37 °C with either 1 mM Gd−DTPA−BAG or 50 mM Gd−HPDO3A. At the end of the incubation, the medium was removed, and the cells were washed three times with ice-cold PBS. Then cells were harvested with a rubber policeman in 250 $\mu$L of PBS and centrifuged at 800*g* for 5 min. The supernatant was discarded, and the cell pellet was resuspended in 100 $\mu$L of $H_2O$ and sonicated for 10 s for a complete lysis of the cells. Untreated cells were incubated, and lysates were collected by following the same procedure.

**MALDI-TOF-MS Experiments.** Each sample was diluted 1:1 with a saturated solution of the matrix (2,4,6-trihydroxyacetophenone, THAP, in 50% acetonitrile), and 0.5 $\mu$L was spotted directly on a MALDI target plate. MALDI mass spectra were acquired in the positive reflectron ion mode with delayed extraction on a Reflex III time-of-flight instrument (Bruker Daltonics, Bremen, Germany) equipped with a 337-nm nitrogen laser. Experimental settings: ion acceleration voltage, 20.00 kV; reflector voltage, 23.00 kV; and first extraction plate, 17.00 kV. Mass spectra were


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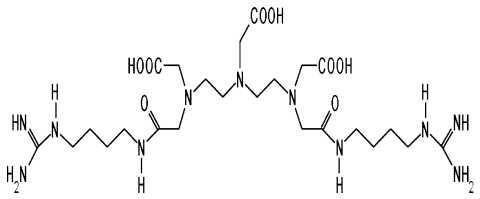

Figure 1. Formula of DTPA−BAG ligand.

obtained by averaging 300 laser shots. Calibration of the spectra was performed externally with a solution of standard peptides covering the range 200−2000 *m*/*z*.

**Quantification by MALDI-TOF-MS.** The standard curve for Gd−DTPA−BAG quantification was obtained by adding to a lysate of untreated HTC cells aliquots of the internal standard Yb−DTPA−BAG (up to a final concentration of 40 $\mu$M) and aliquots of Gd−DTPA−BAG to final concentrations ranging from 4 to 100 $\mu$M (six points, each in triplicate). The same amount of internal standard was then added to unknown samples (deriving from Gd−DTPA−BAG-treated cells), before mixing them to the matrix solution, as previously described.

Peak integration was executed with the X-Mass software (Bruker Daltonics, Bremen, Germany). The ratio of the Gd−DTPA−BAG to Yb−DTPA−BAG signal intensities was measured as the area of the first six monoisotopic peaks of the Gd complex divided by the area of the first six monoisotopic peaks of the Yb complex. This ratio was plotted as a function of the final concentration of Gd−DTPA−BAG in cell lysates.

The same procedure was applied for Gd−HPDO3A. The concentration of the internal standard (the Yb complex) was 400 $\mu$M, and the covered range of the analyte concentration (the Gd complex) was 100 $\mu$M to 1 mM.

## RESULTS AND DISCUSSION

To assess whether it is possible to quantify metal complexes in cell lysates, we first selected a complex, Gd−DTPA−BAG (whose ligand formula is presented in Figure 1), which is positively charged in the lysis conditions (pH around 7−8), as cationic species are likely to give more intense peaks when analyzed with MALDI-TOF-MS in the positive ion mode.

First, it was necessary to find proper experimental conditions which avoid any leakage of the metal during the mass spectrometric measurement. The experiment with α-cyano-4-hydroxycinnamic acid (CHCA) as matrix[16] yielded a MALDI spectrum which shows, in addition to the monocharged peak of the complex, a peak corresponding to the free ligand. This finding clearly indicates a partial dissociation of the complex (Figure 2).

Then 6-aza-2-thiothymine (6-ATT), a neutral matrix often used for noncovalent interactions studies,[17] was tested. This compound does not affect the integrity of the complex but displays a high sensitivity to sample contaminants when the experiment is carried out in a HTC lysate.

Finally, a matrix consisting of a compound often used for oligosaccharides[18] and oligonucleotides[19] analysis, THAP in 50% acetonitrile, gave the best result, because it was possible to find the intact complex peaks also when Gd−DTPA−BAG was dissolved in cell lysates in low micromolar concentrations, while the ligand peak was completely absent (Figure 3).

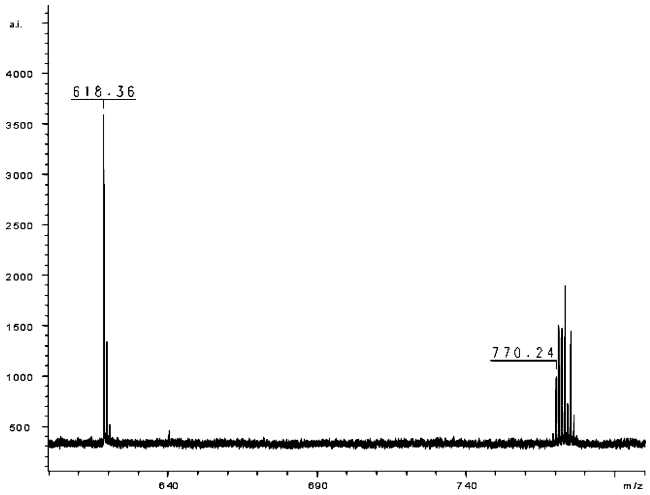


Figure 2. MALDI-TOF spectrum of 40 $\mu$M Gd−DTPA−BAG dissolved in water and mixed with α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile as matrix. The two peaks are the monoprotonated complex (monoisotopic peak at 770 *m*/*z*) and the monoprotonated ligand (monoisotopic peak at 618 *m*/*z*), respectively.

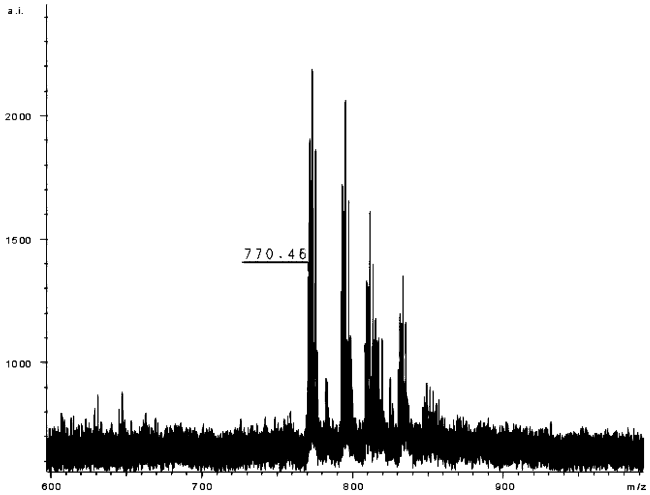


Figure 3. MALDI-TOF spectrum of 40 $\mu$M Gd−DTPA−BAG dissolved in HTC lysate and mixed with 2,4,6-trihydroxyacetophenone in 50% acetonitrile as matrix. The monoprotonated complex peak (monoisotopic peak at 770 *m*/*z*) is present, together with various sodium and potassium adducts, while the monoprotonated ligand (monoisotopic peak at 618 *m*/*z*) is absent.

As the internal standard, a complex with the same ligand but a different metal was used. Metal choice was limited to lanthanides to minimize the differences with the Gd complex for their chemical and physical properties. Initially, we tried with the europium complex, but there were problems concerning both the overlap with Gd-complex peaks (mass difference between the two lanthanides is only 4 Da) and the linearity of the response when quantitative trials were carried out.

Better results were obtained with ytterbium, a lanthanide ion with an isotopic distribution quite similar to that of gadolinium. Their mass difference (16 Da) is enough to avoid overlaps between

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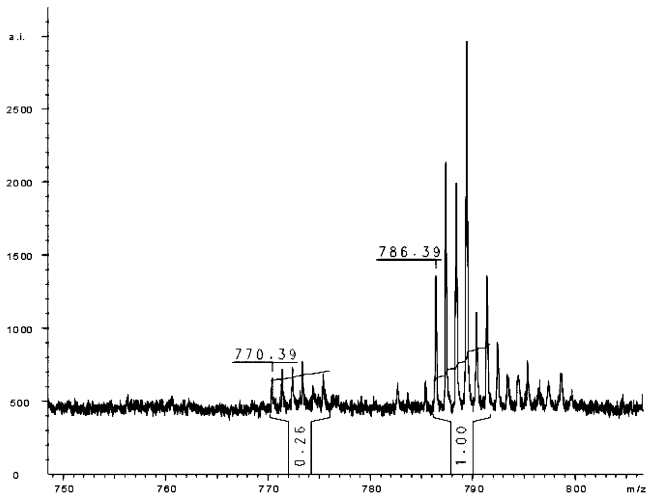


Figure 4. MALDI-TOF spectrum of a lysate from HTC cells treated with 1 mM Gd−DTPA−BAG. The integrations of the monoprotonated peaks of both the analyte (monoisotopic peak at 770 *m*/*z*) and the added internal standard (40 $\mu$M Yb−DTPA−BAG, monoisotopic peak at 786 *m*/*z*) are shown. From the calibration curve, the resulting concentration of the Gd−DTPA−BAG in the HTC lysate was 9 $\mu$M.

standard and analyte peaks (except a negligible overlap between Yb complex and the sodium adduct of the Gd complex).

On this basis, a calibration curve for Gd−DTPA−BAG in HTC lysate was set up by MALDI-TOF-MS. It is usually recommended[11] that a ratio of internal standard to analyte range between 4:1 and 1:4. For a Gd−DTPA−BAG concentration of 4−100 $\mu$M, we used Yb−DTPA−BAG with a concentration of 40 $\mu$M.

To get a stable ratio among the peaks,[12] 300 shots were accumulated for every spectrum. The integration was carried out on the first six monoisotopic peaks of each compound because they are the most intense. Peaks corresponding to sodium and potassium adducts appeared to be a constant percentage if compared to the corresponding monoprotonated peaks and were not considered in the integration procedure.

The best fit line of the calibration curve yields to the equation $y = 0.0298x - 0.0211$ (with a correlation coefficient $R^2 = 0.9967$), where $y$ corresponds to the Gd−/Yb−DTPA−BAG ratio and $x$ is the concentration of Gd−DTPA−BAG in cell lysates. The equation was then used to quantify this complex in unknown samples after treatment of HTC cells with Gd−DTPA−BAG (an example is given in Figure 4, in which the resulting complex concentration was 9 $\mu$M). Because the experimental workup yields a dilution of about 1:20 of the cell content during lysis procedures, the complex concentrations in the cells can be assessed as 20-fold higher than those calculated from the quantification procedure performed on the lysates.

The same procedure was applied for the quantitative determination of Gd−HPDO3A (whose ligand formula is shown in Figure 5), a complex which is neutral in the lysis conditions. The net charge of the complex is expected to affect the quantification assay. In fact, the sensitivity resulted significantly lower than that found with Gd−DTPA−BAG experiments. The lower ionization efficiency is not evident when the complex is analyzed in water (where it is detectable to at least 40 $\mu$M final concentration), but it decreases by 1−2 orders of magnitude when the experiments are performed in cell lysates. No improvement was obtained when negative ions were analyzed.

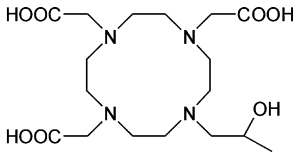


Figure 5. Formula of HPDO3A ligand.

For these reasons, the calibration curve for Gd−HPDO3A covers the range 100 $\mu$M to 1mM, with Yb−HPDO3A 400 $\mu$M as the internal standard.

In the experiments with HPDO3A as the ligand, no peak corresponding to the monoprotonated complex was detectable, whereas the typical gadolinium and ytterbium isotopic distribution pattern was detected for sodium and potassium adducts of the two complexes. Unfortunately, the mass difference between ytterbium and gadolinium (16 Da) corresponds to the mass difference between potassium and sodium, thus yielding to a perfect overlap of potassium adducts of Gd complex with sodium adducts of Yb-one. Nevertheless, choosing the dipotassium adduct of Yb−HPDO3A (which cannot overlap with any Gd−HPDO3A sodium adduct) as internal standard for the calibration curve, we were able to quantify Gd−HPDO3A as its dipotassium adduct. In fact, even if this adduct has the same mass of the sodium−potassium Yb−HPDO3A adduct, the contribution of the latter can be considered constant, because the good linear correlation found demonstrates that in fixed experimental conditions (especially for what concerning cell line and culture medium choice), the partition of the complexes in the various salt adducts is always the same. Interestingly, the disodium, sodium−potassium, and dipotassium adducts appear in the spectrum as monopositively charged peaks, even if they should be doubly charged. This observation can be explained by the (partial) dissociation of the alcohol group present in HPDO3A complexes. In solution, this dissociation occurs only at basic pH[20] ($pK_a = 11.36$ for Ln−HPDO3A);[21] however, it is known that practically, only singly charged ions are detected in MALDI spectra, which are "irrespective of acid−base chemistry in the solution and the charge state of the preformed ion within the matrix−analyte solid".[22]

The best fit for the calibration curve for Gd−HPDO3A corresponds to the equation $y = 0.0069x + 1.8533$ (with a correlation coefficient $R^2 = 0.9953$), where $y$ corresponds to the Gd−/Yb−HPDO3A ratio and $x$ is the concentration of Gd−HPDO3A in cell lysates.

Even with lower sensitivity for the HPDO3A complexes, the calibration curve covers a concentration range that includes the quantities of the complex that were found in the lysates of cells treated with this complex (Figure 6, in which the resulting complex concentration was 160 $\mu$M).

With complexes that are negatively charged under lysis conditions, sensitivity was found to be too low for quantification purposes (data not shown) when both positive and negative ions were analyzed. It has already been reported[22] that signals of negative ion MALDI spectra are markedly lower if compared to positive ones. This fact was more evident in reflectron than in

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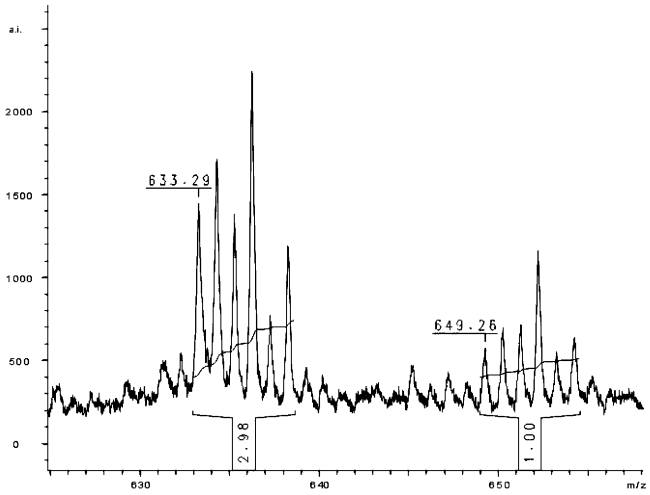


Figure 6. MALDI-TOF spectrum of a lysate from HTC cells treated with 50 mM Gd−HPDO3A. The integrations of the dipotassium adduct peaks of both the analyte (monoisotopic peak at 633 *m*/*z*) and the added internal standard (400 $\mu$M Yb−HPDO3A, monoisotopic peak at 649 *m*/*z*) are shown. From the calibration curve, the resulting concentration of the Gd−HPDO3A in the HTC lysate was 160 $\mu$M.

linear mode, but in the latter case, the quantification was prevented by the worse resolution.

## CONCLUSIONS

For the first time, a quantification of metal complexes has been performed by MALDI-TOF-MS. Conditions have been optimized, especially concerning the matrix, for detection in cell lysates without loss of the ion from the complex. 2,4,6-Trihydroxyacetophenone in 50% acetonitrile was shown to be the best matrix for this purpose. With this approach, no pretreatment is needed, and the specificity of the technique allows discrimination between Gd complexes and other gadolinium-containing compounds.

In cell lysates, neutral complexes showed a lower ionization efficiency when compared to cationic ones. The lower limit of detection of both cationic and neutral complexes (4 and 100 $\mu$M, respectively) was shown, however, to be enough for the quantification of these complexes in treated cells, as proved by uptake experiments. Sensitivity was still lower for anionic compounds, preventing in this case the setup of a calibration curve in a reasonable range of concentrations. The good linear correlation found for the calibration curves demonstrates that a metal complex with the same ligand of the analyte can be considered as good as a stable isotope-labeled compound, even considering the variability in sample/matrix cocrystallization and desorption/ionization processes. We think that this approach for the quantification can be extended to many kinds of metal complexes, at least to cationic or neutral ones. Upon choosing the chemical equivalent complex as an internal standard, it is particularly important to first inspect the similarity of chemical properties, physical properties, and isotopic distribution with respect to the analyte.

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