Education and Culture DG
Lifelong Learning Programme

Intensive programme
Design, Synthesis and Validation of Imaging Probes
Turin (Italy) – September 19 to 30, 2011

Imaging Probe: definition and design

Alessandro Barge

Dipartimento di Scienza e Tecnologia del Farmaco,
Università degli Studi di Torino

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Molecular Imaging Contrast Agents

Activity

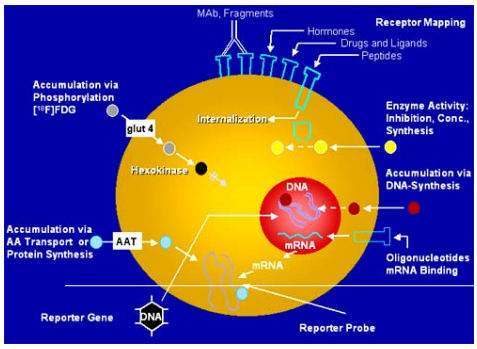
Targeted

Responsive (SMART)

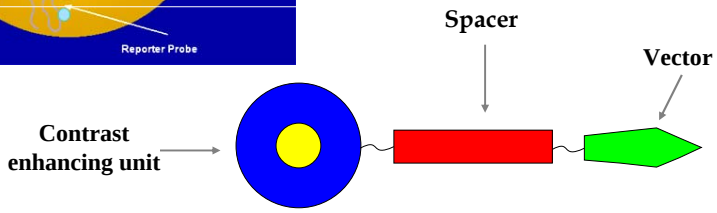
Imaging Technique

- Optical imaging
- *PET*
- *Spect*
- *MRI*
- US

Targeted Molecular Imaging CAs



Molecular imaging contrast agent



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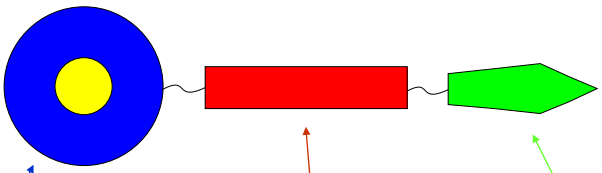
Targeted Molecular Imaging CAs



Contrast enhancing unit

- ✓**High thermodynamic stability (***especially when it is a metal complex***)**
- ✓**High contrast efficiency**

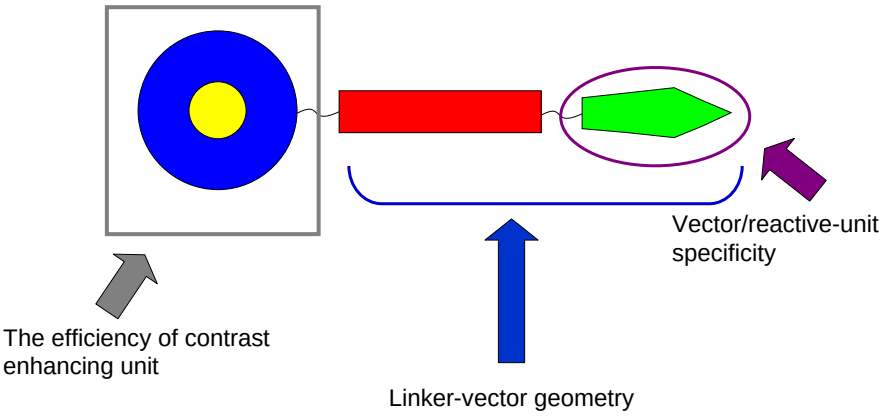
Spacer

- ✓**Linear and flexible**
- ✓**Linear and rigid**
- ✓**Cyclic**
- ✓**Aromatic**

Vector

- ✓**Small organic molecule**
- ✓**Peptide**
- ✓**Peptidomimetic**
- ✓**Protein**

To increase sensitivity and enhance tissue, cellular, or molecular specificity it has to be optimized:



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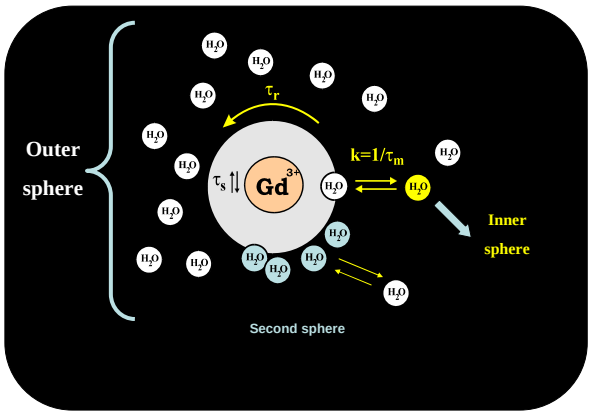
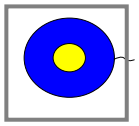
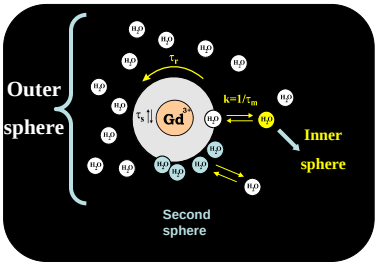
MRI CAs relaxivity parameters



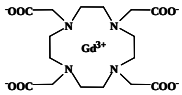
- Inner sphere water molecules ***q***
- Second sphere water molecules ***n***
- Water exchange lifetime τ_M
- Reorientation correlation time τ_R



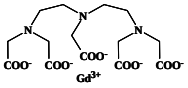


Optimization of efficiency of contrast enhancing unit

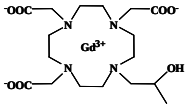
Commercially available Contrast Agents



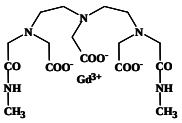
[Gd-DOTA]⁻
DOTAREM® (Guerbet)



[Gd-DTPA]²⁻
MAGNEVIST® (Schering)



[Gd-HPDO3A]
PROHANCE® (Bracco)



[Gd-DTPABMA]
OMNISCAN® (Nycomed)

Inner sphere water molecules = 1

Second sphere water molecule = 0

τ_M and τ_R not optimal

Modest efficiency

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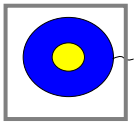
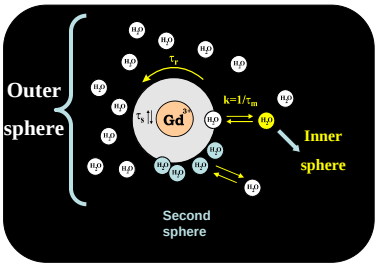
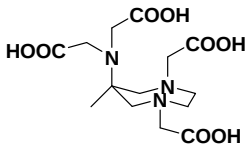




Optimization of efficiency of contrast enhancing unit

1. ***Increase q by changing the coordination cage***



GdAAZTA

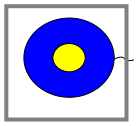
2 inner sphere water molecule

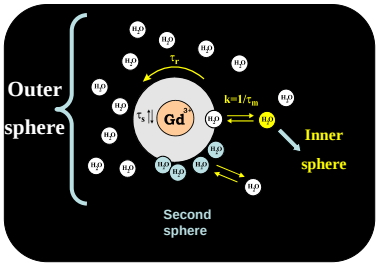
More efficient contrast agent

Develop new contrast agents based on

6-amino-perhydro-1,4-diazepine scaffold

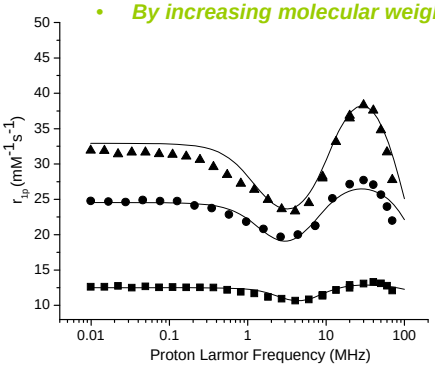
Optimization of efficiency of contrast enhancing unit





2. *Reducing molecular tumbling*

- *By increasing molecular weight*



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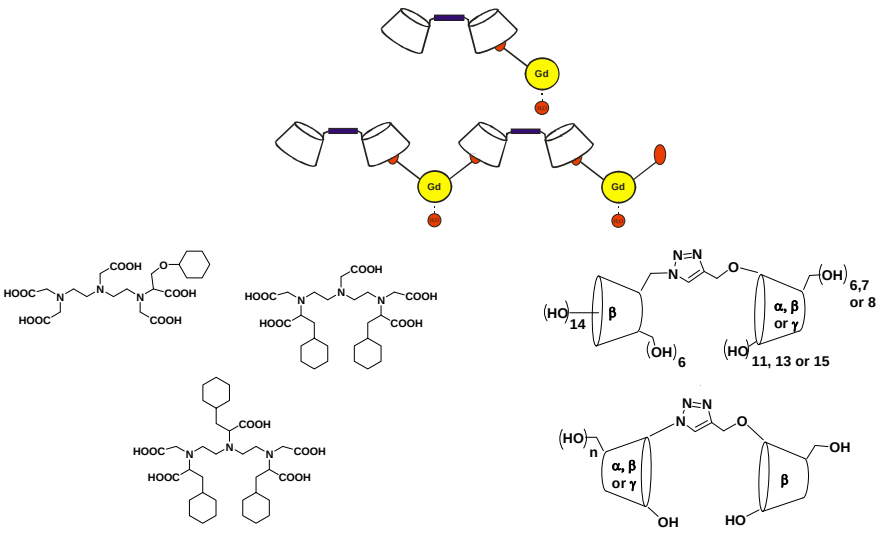
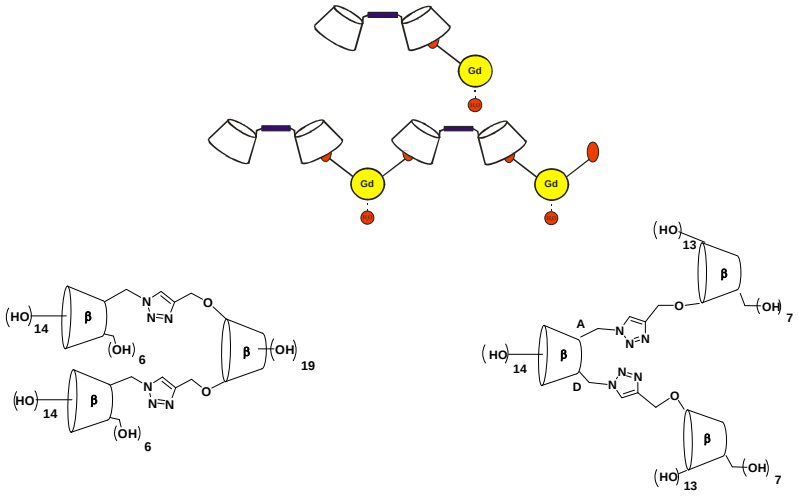


Cravotto, G.; Mendicuti, F.; Martina, K.; Tagliapietra, S.; Robaldo, B.; Barge, A. *Synlett* **2008**, *18*, 2642-2646



Barge, A.; Aime, S.; Gianolio, E.; Martina, K.; Cravotto, G.; *Org. Biomol. Chem.*, **2009**, **DOI:** 10.1039/b812172a

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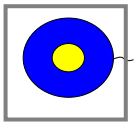
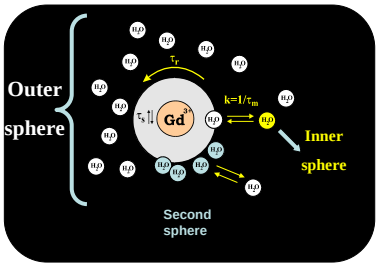
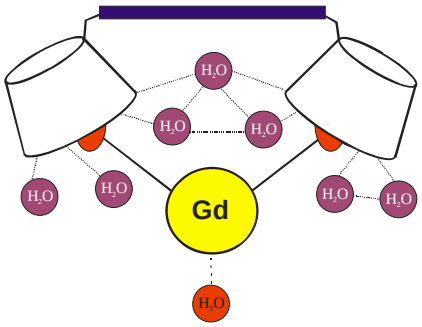
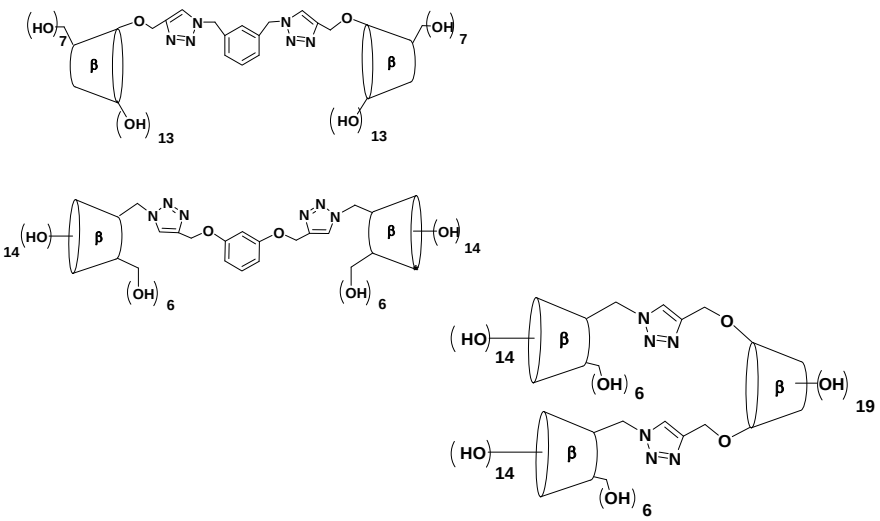




Optimization of efficiency of contrast enhancing unit

2. ***Reducing molecule tumbling***
   - ***By increasing molecular weight***
3. ***Including the second coordination sphere contribution***





Barge, A.; Aime, S.; Gianolio, E.; Martina, K.; Cravotto, G.; *Org. Biomol. Chem.*, **2009**, **DOI:** 10.1039/b812172a

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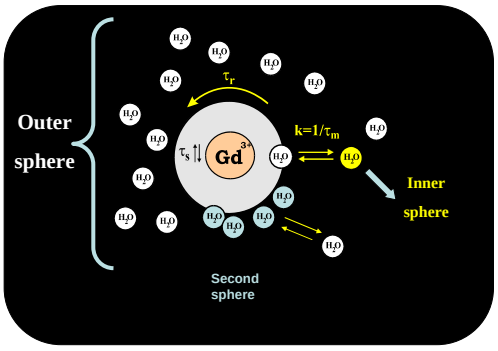
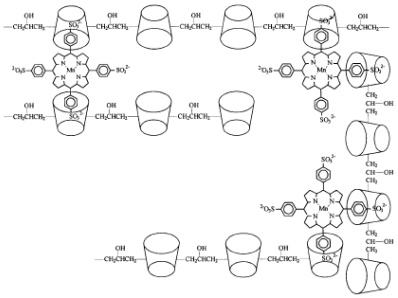
SMART contrast agents

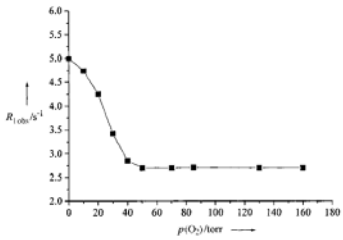


Angew. Chem. Int. Ed. 2000, 39, No. 4, 747

A $p(O_2)$-Responsive MRI Contrast Agent Based on the Redox Switch of Manganese(II/III) – Porphyrin Complexes

Silvio Aime,* Mauro Botta, Eliana Gianolio, and Enzo Terreno

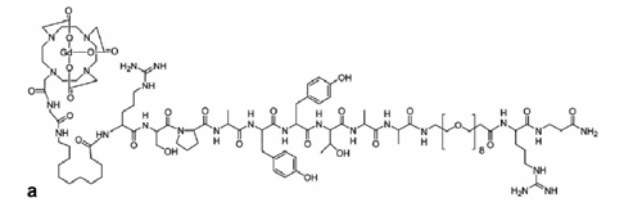




Magnetic Resonance in Medicine 60:1056–1065 (2008)

Novel Solubility-Switchable MRI Agent Allows the Noninvasive Detection of Matrix Metalloproteinase-2 Activity In Vivo in a Mouse Model

Réjean Lebel,[1] Beata Jastrzębska,[1] Hélène Therriault,[2] Marie-Michèle Cournoyer,[1] J. Oliver McIntyre,[3] Emanuel Escher,[4] Witold Neugebauer,[4] Benoit Paquette,[2] and Martin Lepage[1]*

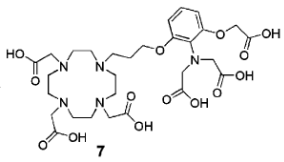


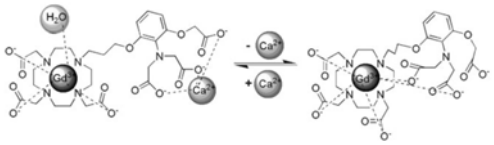
COMMUNICATION www.rsc.org/chemcomm | ChemComm

Synthesis and characterization of a smart contrast agent sensitive to calcium†

Kirti Dhingra,*[a] Martin E. Maier,[b] Michael Beyerlein,[a] Goran Angelovski[a] and Nikos K. Logothetis*[ac]

Received (in Cambridge, UK) 4th February 2008, Accepted 25th April 2008
First published as an Advance Article on the web 4th June 2008
DOI: 10.1039/b801975d



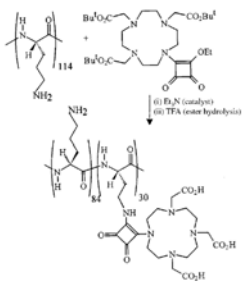


Chem. Commun., 1999, 1577–1578

ChemComm

A macromolecular Gd(III) complex as pH-responsive relaxometric probe for MRI applications

Silvio Aime,*[a] Mauro Botta,[b] Simonetta Geninatti Crich,[a] Giovanni Giovenzana,[c] Giovanni Palmisano[d] and Massimo Sisti[e]



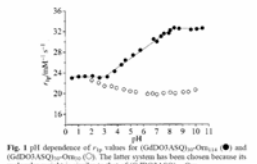


Fig. 1 pH dependence of r_{1p} values for (GdDO3ASQ)$_{30}$-Orn$_{114}$ (●) and (GdDO3ASQ)$_{30}$-Orn$_{30}$ (○). The latter system has been chosen because its molecular weight is similar to that of (GdDO3ASQ)$_{30}$-Orn$_{114}$.

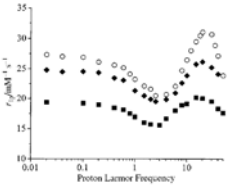


Fig. 2 $1/T_1$ NMRD profiles (298 K) of (GdDO3ASQ)$_{30}$-Orn$_{114}$ (1 mM) at pH 4.5 (■), 7 (◆) and 8.5 (□) respectively.

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About MRI contrast enhancing unit… ligands for Gd(III) ion

DSTF Dipartimento di Scienza e Tecnologia del Farmaco UNIVERSITÀ DI TORINO

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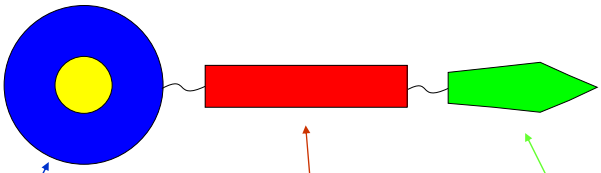
MR Molecular imaging contrast agent



Contrast enhancing unit

✓**High thermodynamic stability**

✓High contrast efficiency

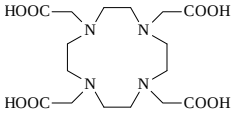
Spacer

✓Linear and flexible
✓Linear and rigid
✓Cyclic
✓Aromatic

Vector

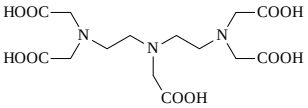
✓Small organic molecule
✓Peptide
✓Peptidomimetic
✓Protein

Recommended ligand types



DOTA

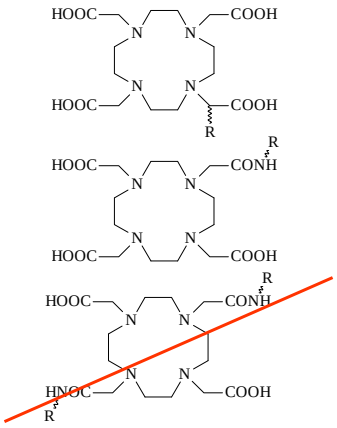
-Cyclic ligand
-Octadentate
-Forms very stable Gd Complexes
$K_{GdL} = 10^{24}$



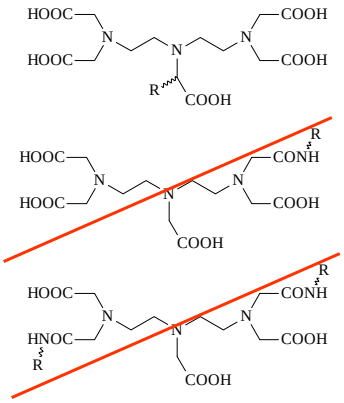
DTPA

-Linear ligand
-Octadentate
-Forms very stable Gd Complexes
$K_{GdL} = 10^{22}$

Recommended ligand modification

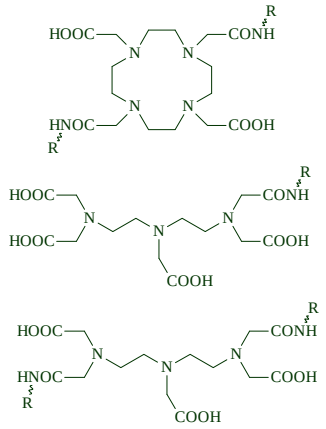


DOTA - Like



DTPA - Like

Why not DOTAbisamides and DTPAmono and bisamides?



Increase of τ_M

Decrease of complex stability constant

Increasing in toxicity due to the free metal ion

Alter the *in-vitro* and *in-vivo* cellular uptake experiments

Contrast Med. Mol. Imaging, 2006, **1**, 23-29

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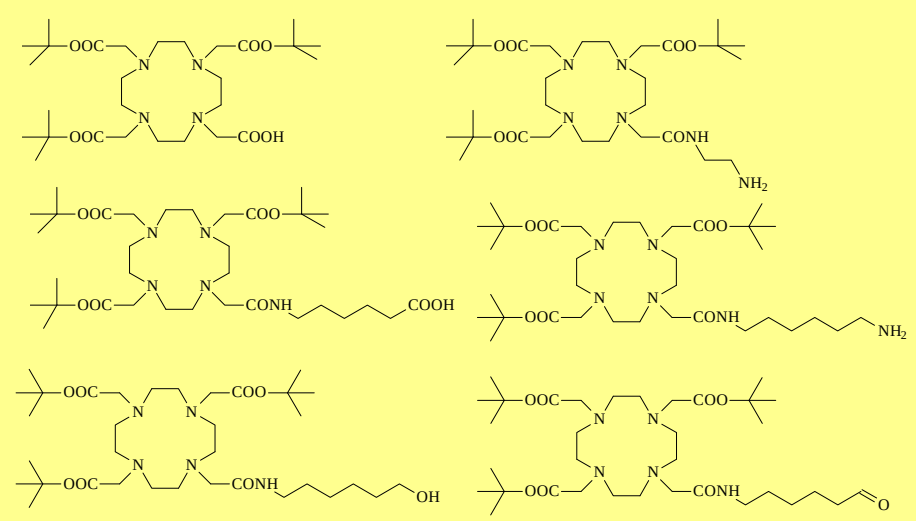
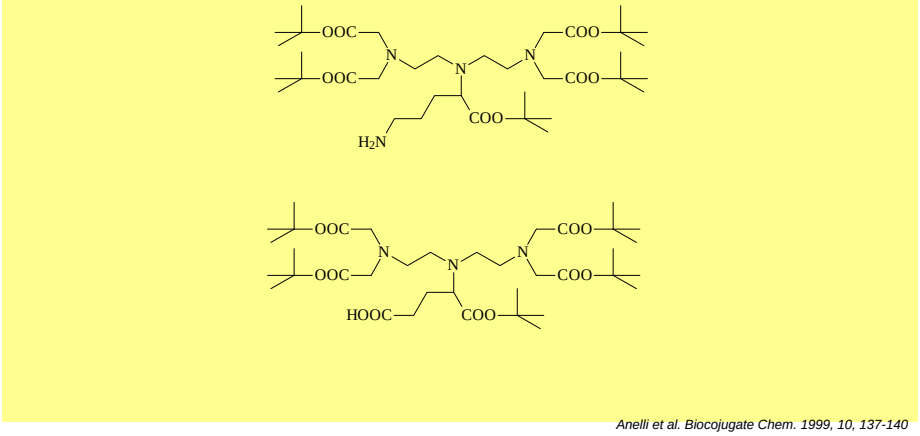
Available intermediates



A. Barge, L. Tei, S. Aime, et al. Org. Biomol. Chem., 2008, **6**, 1176–1184

Available intermediates



Anelli et al. Biocojugate Chem. 1999, 10, 137-140

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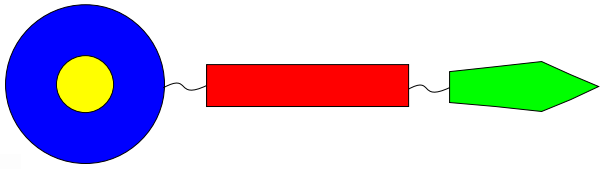
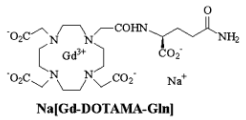
Spacer/linker…
… its role



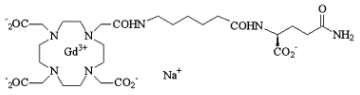
4926 *J. Med. Chem.* **2006,** *49,* 4926−4936

In Vitro and in Vivo Magnetic Resonance Detection of Tumor Cells by Targeting Glutamine Transporters with Gd-Based Probes

Simonetta Geninatti Crich,[†,||] Claudia Cabella,[‡] Alessandro Barge,[§,||] Simona Belfiore,[†,||] Cristina Ghirelli,[‡] Luciano Lattuada,[#] Stefania Lanzardo,[⊥] Armando Mortillaro,[†,||] Lorenzo Tei,[†,||] Massimo Visigalli,[‡] Guido Forni,[⊥] and Silvio Aime*[,†,||]



Na[Gd-DOTAMA-Gln]



Na[Gd-DOTAMA-C_6-Gln]

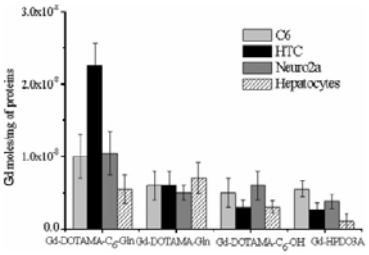


Figure 1. Internalization of Gd chelates in different cell types upon incubation for 6 h at 37 °C in the presence of 1.6 mM of the MR-imaging probes.

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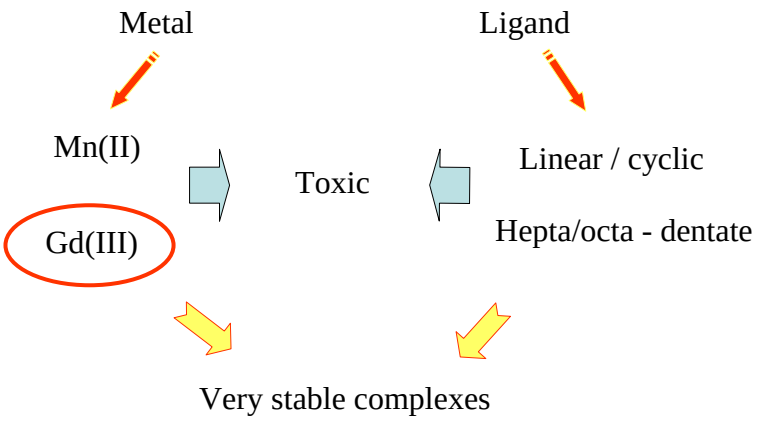
Preparation and Analysis of Gd Complexes



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Synthesis of the desired ligand, final product requirements:

- High purity
- Complete characterization of the ligand
- Complete characterization of residual impurity
- Quantification of the residual impurity (if possible)

The impurity eventually present in the ligand preparation can play a fundamental role in increasing global toxicity of the final formulation because

•They are toxic

•They form labile metal complexes that increase the overall concentration of free metal in physiological conditions

WARNING
Formation of labile metal complexes can alter the in-vitro experiments especially cellular uptake experiments.

Contrast Med. Mol. Imaging, 2006, **1**, 23-29

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Characterization of the final ligand

•HPLC analysis

•Complete NMR characterization

•Mass analysis

•Acidimetric titration

•Complexometric titration

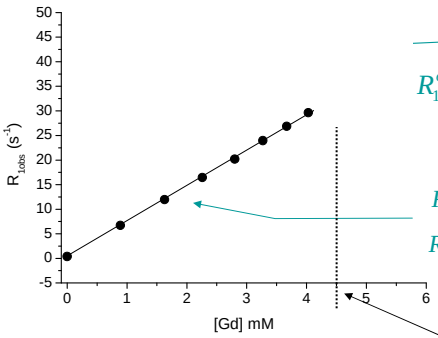
Complexometric titration followed by relaxometry

$$R_1^{obs} = R_1^{dia} + r_{1p}^{GdL}[GdL] + r_{1p}^{Gd^{3+}}[Gd^{3+}_{free}]$$

$$R_1^{obs} = R_1^{dia} + r_{1p}^{GdL}[L] + r_{1p}^{Gd^{3+}}([Gd^{3+}] - [L])$$

$$R_1^{obs} = R_1^{dia} + r_{1p}^{GdL}[GdL]$$

$$R_1^{obs} = R_1^{dia} + r_{1p}^{GdL}[Gd^{3+}]$$



Equivalent point

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Complexometric titration followed by relaxometry

- It is easy to do
- It can applicable only on fast complexation reaction
- The measure is done on Gd^{3+}

Complexometric titration with ZnSO4

- It is a simple, classical, complexometric titration
- No kinetic problems
- But it does not use a Ln ion

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Complex synthesis

- Kinetic aspects play a fundamental role in metal complexation process
  - Type of coordination cage (DOTA or DTPA like)
  - Role of modified added arm
- Formation of labile complexes between metal and coordinating groups eventually present on the surface of the added arm

Complex preparation strategies

- Using Ln_2O_3
- Using $Ln(OH)_3$
- Using $LnCl_3$
- Using Ln(NTA) or LN(EDDA)

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Complex preparation strategy 1

Using Ln_2O_3

- It allows to obtain a complex preparation free of salts
- It needs to operate at 100°C – it is not indicate for labile ligands
- Very slow reaction kinetic essentially due to low solubility of Ln_2O_3

Complex preparation strategy 1

Using Ln_2O_3

- Dissolve the ligand in water
- Add a stoichiometric amount of Ln_2O_3 (better if it is in very low excess)
- Eventually add NaOH to complete the ligand neutralization.

  (DOTA needs 1 equivalent of Ln_2O_3 and 1 equivalent of NaOH)
- Stir at reflux conditions for several hours (or several days) until the oxide is solubilized
- Filter the solution and check the free Ln(III) amount by UV-Vis or by titration
- Add a ligand amount equimolar to the Ln(III) excess
- Check the Ln(III) excess and eventually repeat the last two steps

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Complex preparation strategy 2

Using $Ln(OH)_3$

- It allows to obtain a complex preparation free of salts
- It needs to operate between 70 and 100°C
  – it is not indicate for labile ligands -
- Relatively fast kinetic.

Complex preparation strategy 2

Using $Ln(OH)_3$

- Dissolve a desired amount of $LnCl_3$ in water
- Add a NaOH 2M solution allowing the complete hydroxide precipitation
- Centrifuge the mixture and eliminate the supernatant.
- Wash the precipitate with NaOH and than with water until the supernatant reaches neutral pH.
- Dissolve an equimolar amount of ligand (it is better if you use always a very small excess of Ln) in water and with this solution suspend the $Ln(OH)_3$ precipitate.
- Eventually add NaOH to complete the ligand neutralization.

  (DOTA needs 1 equivalent of Ln_2O_3 and 1 equivalent of NaOH)
- Stir at 70 – 100°C until the oxide is solubilized (reaction needs from 2 hours to few days, depending from both metal and ligand)
- Filter the solution and check the free Ln(III) amount by UV-Vis or by titration
- Add a ligand amount equimolar to the Ln(III) excess
- Check the Ln(III) excess and eventually repeat the last two steps

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Complex preparation strategy 3

Using $LnCl_3$

- You introduce a not negligible amount of salts
- It is possible to complex the metal at room temperature
- Relatively fast kinetic.

Complex preparation strategy 3

Using $LnCl_3$

- Dissolve the desired ligand amount in water (It is better to use concentration more than 30 mM)
- Change the pH value to 6 – 6.5 with NaOH
- Slowly add a metal chloride water solution (use a small excess) . Check the pH and restore the previous value
- Stir the solution overnight at RT
- Move the pH to 8 – 8.5 and stir for 2 hours (this procedure allows complete precipitation of metal hydroxide)
- Restore the pH to 6.5 – 7 and determine the residual metal excess by UV method
- Slowly add the ligand water solution (in stoichiometric ratio with free metal or in small excess) . Check the pH and restore the previous value
- Repeat the last four steps

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Complex preparation strategy 4

Using Ln(NTA) or Ln(EDDA)

- It is the only recommended method when you use a ligand linked to a protein surface
- It avoids the possibility to bind the metal ion to protein
- Relatively slow kinetic.

Complex preparation strategy 4

Using Ln(NTA) or Ln(EDDA)

- Prepare Ln(L)
- Dissolve the desired amount of ligand in a TRIS buffered solution (pH=7)
- Add Ln(L) to ligand solution (use ten fold excess)
- Stir the solution overnight at 4°C
- Add 2 equivalents of Na_nL and stir at RT for 2 hours
- Purify the complex by Size Exclusion Chromatography
- Check the amount of free Ln(III) eventually present and add Na_nL (two fold excess)
- Repeat the last two steps

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Free Gd(III) is a dangerous impurity in the probe preparation for MR Molecular Imaging Applications.

It can significantly alter the *in vivo* or *in vitro* experimental results.

Its concentration has to be lower than 0.3% (mol/mol)

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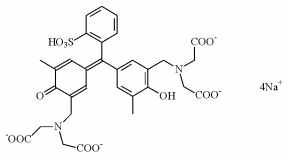
Uv-Vis method used to quantify free Ln(III) in Ln complex solution

Xilenol Orange solution (buffered at pH=5.8) change its color depending on the free Ln(III) concentration



The iminodiacetic and oxydrile groups coordinate metal ions causing phenol deprotonation and consequent extension of electronic delocalization

NOTE: this method is tested only on Gd(III) complex solution
log $K_{(Gd\text{-}Xylenol\ Orange)}$=5.8

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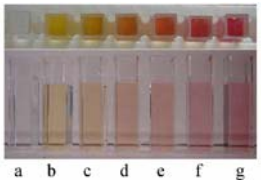
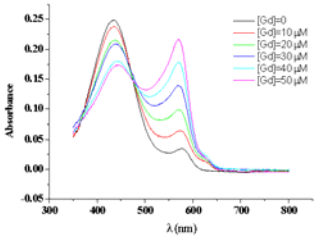
Uv-Vis method used to quantify free Ln(III) in Ln complex solution

The formation of metal complex causes a change in the color solution from yellow to violet



The absorption band at 433 nm decrease while the band at 573 nm increase depending on the Gd(III) concentration



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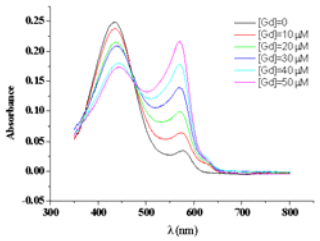
Uv-Vis method used to quantify free Ln(III) in Ln complex solution

The free Gd(III) concentration is directly proportional to the ratio of the absorbances at 573 and 433 nm. Over a given concentration range of free Gd(III) a linear dependence is observed.

$$[Gd^{3+}] = A + B\frac{Abs^{573}}{Abs^{433}}$$



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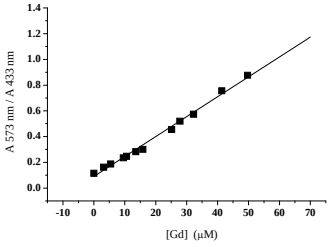
Uv-Vis method used to quantify free Ln(III) in Ln complex solution

As for all spectrophotometric methods, also in this case it is essential to standardize the measure by the use of a set of Gd(III) solutions covering a well defined concentration range.



$$[Gd^{3+}] = A + B\frac{Abs^{573}}{Abs^{433}}$$

Gd concentration is referred to standard before dilution with dye

Dye solution preparation and method calibration

•Dissolve 3.0 mg of Xylenol Orange in 250 ml of Acetic buffer 10mM at pH = 5.80.

•Divide the solution into many single-use 600 μl portions and freeze them

Thaw a dye vial

Put 500 μl of dye in a microcuvette and measure the wavelengths ratio

$$ratio = \frac{Abs \quad 573nm}{Abs \quad 433nm}$$

Add 50 μl of Gd^{3+} standard solution to dye and shake

Measure the wavelengths ratio

Draw the calibration curve

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Sample measurement

Thaw a dye vial

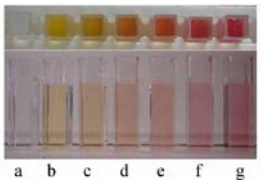
Put 500 μl of dye in a microcuvette and measure the wavelengths ratio

$$ratio = \frac{Abs \quad 573nm}{Abs \quad 433nm}$$

Add 50 μl of complex solution to dye and shake

Measure the wavelengths ratio

Calculate the Gd(III) free concentration using the calibration curve



NOTE: the best results are obtained using sample solution concentration more than 35 mM

Complexometric method used to quantify free Ln(III) in Ln complex solution

Preparation of acetic buffer at pH 5.8.
Acetic acid (5.75 ml) is dissolved in water (700 ml) and the pH is corrected to 5.8 with NaOH. Finally the volume of the solution is adjusted to 1 litre with distilled water.

Preparation of Xylenol Orange solution.
Xylenol orange (30 mg) is dissolved in acetic buffer at pH 5.8 (100 ml). The obtained solution has to be stored in the dark at 48C for not more than one week.

Preparation of EDTA 0.001M solution.
10.00 ml of Titriplex, 0.1 mol/l, are diluted to 1.00 litre with water.

Preparation of the 0.001 M solution of Gd^{3+} ions.
Gadolinium chloride (3.72 g) is dissolved in water (100 ml); 10 ml of this solution are then diluted to 1 litre with water. The concentration of the final solution is determined by complexometric titration.

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Complexometric method used to quantify free Ln(III) in Ln complex solution

weigh an aliquot of the complex (containing at least $3x10^5$ equivalents) and dissolves it in acetic buffer (10 ml) and water (20 ml).

Add few drops of orange xylenol (the solution become violet)

The solution is then titrated with EDTA 0.001 M by a burette in steps of 0.05 ml until the solution color switches from violet to yellow

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Complexometric method used to quantify free ligand in Ln complex solution

weigh an aliquot of the complex (containing at least $3x10^5$ equivalents) and dissolves it in acetic buffer (10 ml) and water (20 ml).

Add few drops of orange xylenol (the solution become yellow)

During this titration the occurrence of slow kinetics of the complexation reaction has to be taken into account. In this case it is necessary to wait enough time after changes in the indicator's colour in order to be sure that the reaction is complete.

The solution is then titrated with $GdCl_3$ 0.001 M by a burette in steps of 0.05 ml until the solution color switches from yellow to violet

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Complexometric method used to quantify free ligand in Ln complex solution

Ligands that show a very slow kinetics can be treated as follow

weigh an aliquot of the complex (containing at least $3x10^5$ equivalents) and dissolves it in acetic buffer (10 ml) and water (20 ml).

Add few drops of orange xylenol (the solution become yellow)

Add a known amount of $GdCl_3$ and allow it to be complexed (the solution color change from yellow to violet)

Titrate the Gd(III) excess as previously descipted

Free ligand should be less than 1%, otherwise the complex preparation could be toxic.

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Complex purification

Often salts present in the complex preparation can be a serious problem, because they can drammatically change the solution ionic strength and osmolarity

Osmolarity is an important parameter directly involved in the toxicity of the final preparation

Complex desalting

Size esclusion cromatography

Based on the different molecular size between complex and salts

Sephadex G10
Sephadex G25

Nanofiltration // dialysis

Availability of adequate instrumentation

Use membrane with small cut-off (200 – 500 Da)