Fluorescence-enhanced europium complexes for the assessment of renal function

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ABSTRACT

Real-time, non-invasive assessment of glomerular filtration rate (GFR) is essential not only for monitoring critically ill patients at the bedside, but also for staging and monitoring patients with chronic kidney disease. In our pursuit to develop exogenous luminescent probes for dynamic optical monitoring of GFR, we have prepared and evaluated Eu^{3+} complexes of several diethylenetriamine pentaacetate (DTPA)-monoamide ligands bearing molecular "antennae" to enhance metal fluorescence via the intramolecular ligand-metal fluorescence resonance energy transfer (FRET) process. The results show that Eu-DTPA-monoamide complex **13a**, which contains a quinoxanlinyl antenna, exhibits large (c.a. 2700-fold) Eu^{3+} fluorescence enhancement over Eu-DTPA (**4c**). Indeed, complex **13a** exhibits the highest fluorescent enhancement observed thus far in the DTPA-type metal complexes. The renal clearance profile of the corresponding radioactive ^{111}In complex **13c** is similar to that of ^{111}In-DTPA, albeit **13c** clears slower than ^{111}In-DTPA. The biodistribution data indicates that **13c**, and, by inference, **13a** clear via a complex mechanism that includes glomerular filtration.

Keywords: FRET, GFR, fluorescence enhancement, europium fluorescence, molecular antenna, DTPA, MAG_3.

1. INTRODUCTION

It is well recognized that the degree of glomerular filtration rate (GFR) represents the best overall measure of kidney function in the state of health or illness.[1] In the past three decades, endogenous markers such as creatinine[2] (**1**), and exogenous agents such as inulin[3] (**2**), iothalamate[4] (**3**), and ^{99m}Tc-DTPA,[5] (**4a**) have been developed to determine GFR, but all of them require either radiometric, HPLC, or X-ray fluorescence methods for detection and quantification (Fig. 1).

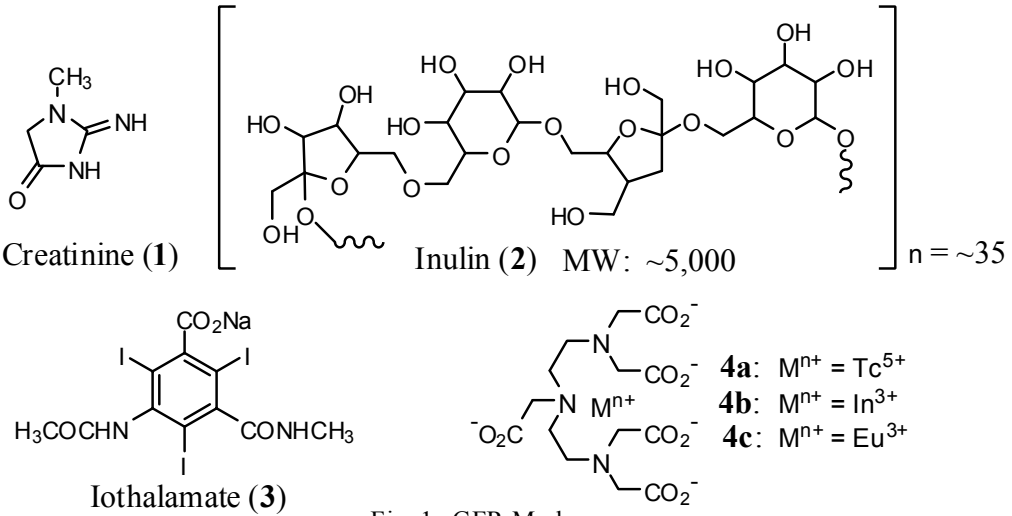

Fig. 1. GFR Markers

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Theoretical methods for estimating GFR from body cell mass and plasma creatinine have also been developed,[6] but these methods depend upon many clinical and anthropometric variables such as age, renal perfusion, muscle mass, etc. that would require some correction to the theoretically estimated GFR value. The availability of non-radioactive exogenous markers that provide rapid, dynamic (i.e. continuous, real-time), and accurate measure of renal excretion rate would represent a substantial improvement over any currently available or widely practiced method. Moreover, since such a method would depend solely on the renal elimination of the exogenous chemical entity, the measurement would be absolute, and would not involve subjective interpretation based on age, muscle mass, blood pressure, etc.

Considerable effort is now being directed at developing exogenous GFR agents for rapid real-time assessment of specific renal function using non-radioactive methods.[7-11] In particular, the effort is focused on luminescent agents that absorb and emit light in the visible or NIR regions. In principle, two general approaches for designing fluorescent renal agents can be considered: the first method involves enhancing the fluorescence of known renal agents that are intrinsically poor emitters such as lanthanide metal complexes; and the second involves transforming highly fluorescent dyes (which are intrinsically lipophilic) into hydrophilic, anionic species to force them to clear via the kidneys.[12] This paper focuses on enhancing the fluorescence via the (FRET) process. The use of molecular 'antennae' to boost the fluorescence of lanthanide ions such as europium, terbium, and dysprosium, has been reported previously,[13-16] and several europium complexes of DTPA ligands endowed with benzopyranone antennae have been shown to enhance europium fluorescence by three orders of magnitude compared to Eu-DTPA.[15] Also, metal complexes such as Gd-DTPA and In-DTPA have been shown to clear via the glomerular filtration pathway.[17-19] As a part of our continuing efforts to develop exogenous fluorescent GFR markers that absorb and emit in the visible region, we wish to report our recent findings on the fluorescence and renal clearance properties of some novel polyaminocarboxylate complexes **5-16** attached to various mono- and binuclear aromatic antennae (Fig. 2).

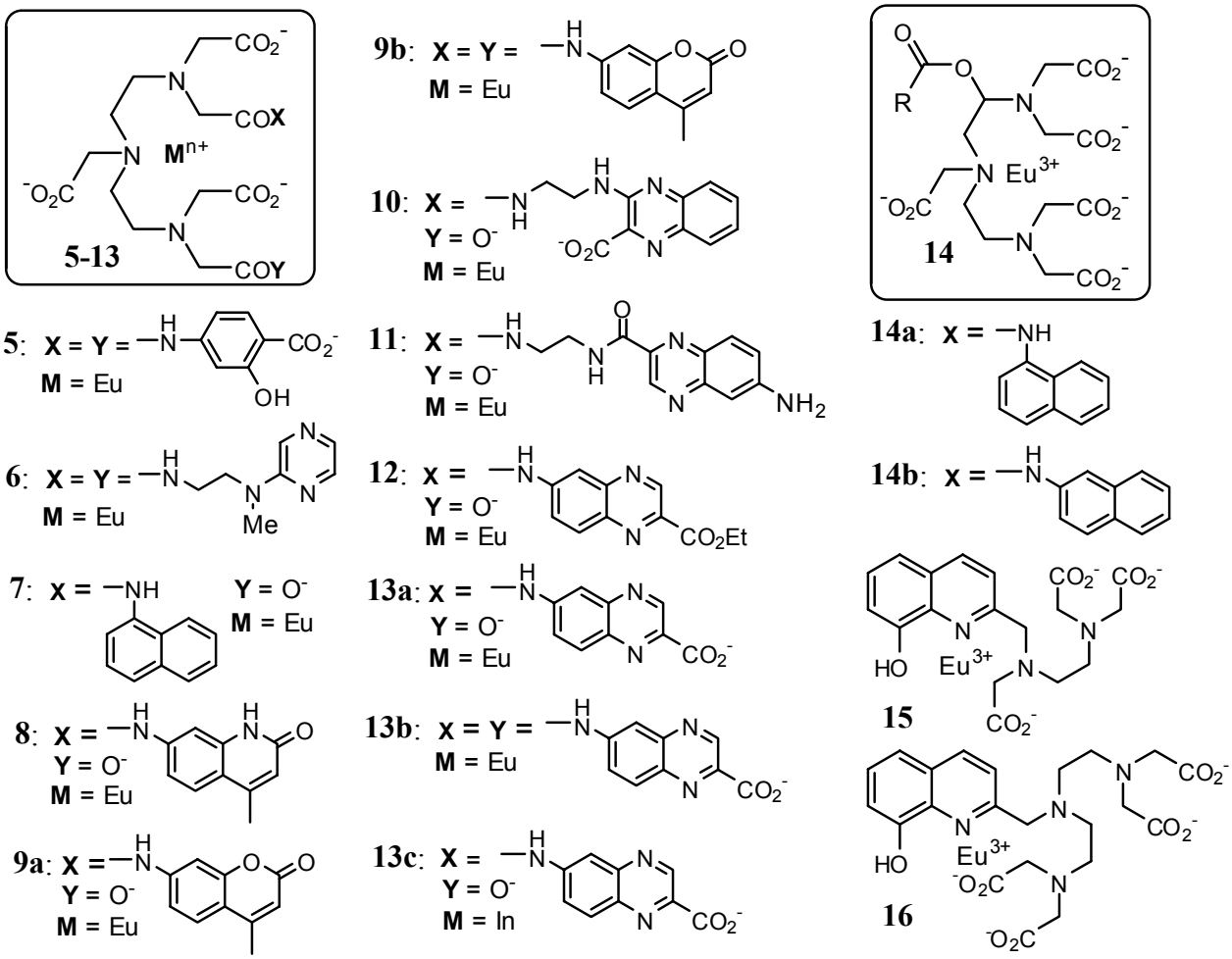


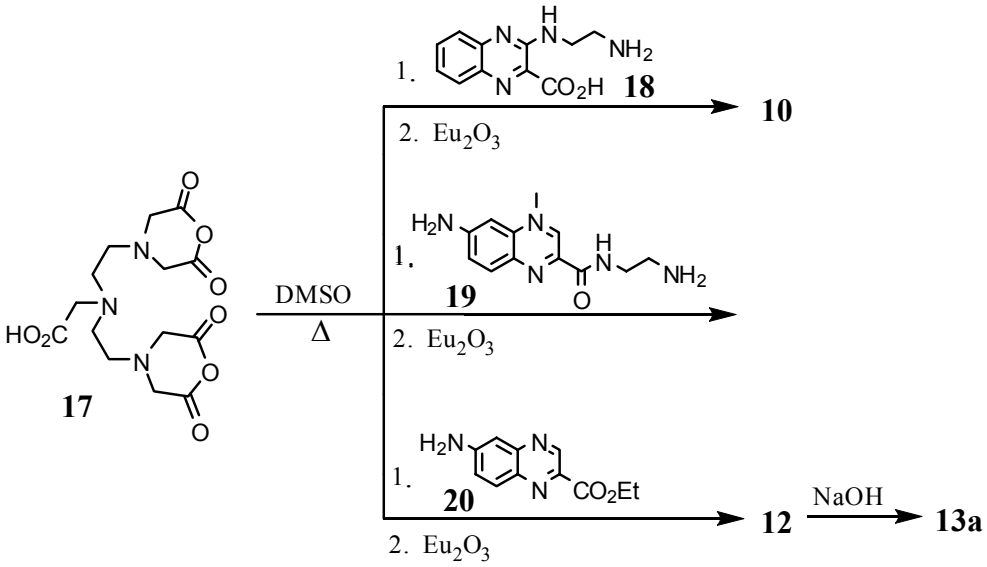
Fig. 2. Metal Complexes Containing Molecular Antenna

2. MATERIALS AND METHODS

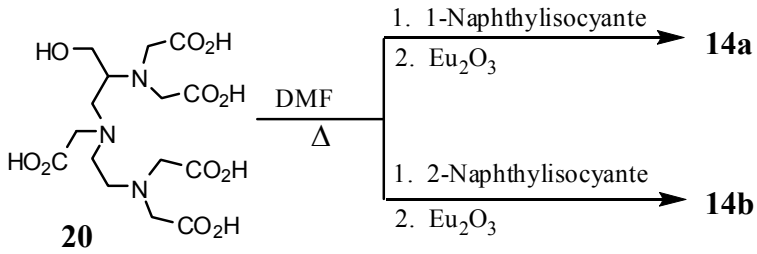
2.1. Preparation of europium complexes.

Preparation and Eu^{3+} complexation of DTPA mono- and bis-amides (**5-13**) were prepared by standard method[15, 20] of reacting DTPA-bis(anhydride) with equivalent amount of desired amines followed by heating the mixture of europium oxide with the respective ligands in water (Scheme 1). Preparation of carbamoyloxymethyl-DTPA derivatives **14a** and **14b** were prepared by the condensation of hydroxymethyl-DTPA[21] with α- or β-naphthylisocyanate respectively, followed by the reaction of the corresponding ligands with europium oxide (Scheme 2).

Scheme 1

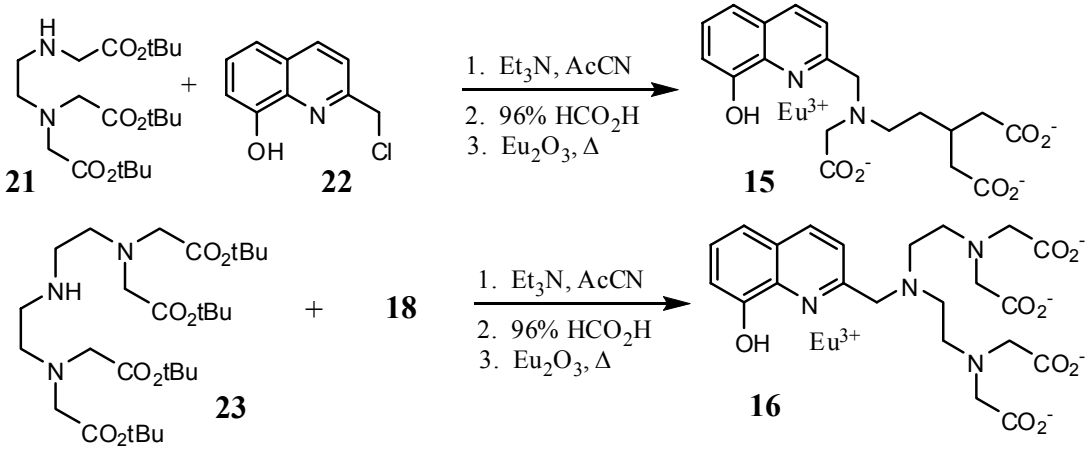


Scheme 2



The EDTA- and DTPA-quinoline complexes **15** and **16** were prepared in three steps (Scheme 3)[16]: alkylation of **21**[22] or **23**[22] with 2-chlormethyl-8-hydroxyquinoline,[23] deprotection of the t-butyl groups; and complexation with europium oxide.

Scheme 3



2.2. Radiolabeling procedure.

^{111}In-labeling of the respective ligands was carried out by known procedures.[24] ^{111}In was selected as the preferred radionuclide for biodistribution studies due to ready availability of ^{111}In in a carrier-free form and ease of complexation, and because ^{111}In-DTPA is known to be a GFR marker.[19] To a 3-mL vial was added 100 µL ligand solution (10 mg/mL in water or 0.05 N NaOH) followed by 250 µL of ^{111}In in 0.05 N HCl (ca. 1 mCi). Next, 10 µL of 0.5 N NaOAc was added, and the resulting solution was allowed to stand at room temperature for 10-15 minutes. Radiochemical purities of the ^{111}In complexes were determined by HPLC using an XTerra C-18 column (4.6 x 250 mm, 5 µ) and 0.1%TFA/acetonitrile gradient. Reaction mixtures were diluted to 125 µCi/mL with 0.9% NaCl for biodistribution studies, and the stabilities of the diluted preparations were evaluated by radiochemical purity assay at six hours post dilution

2.3. Biodistribution protocol.

Sprague-Dawley (Harlan, Indianapolis, IN) rats (n = 3) (170-273 g) were given 25 µCi (0.20 mls of 125 µCi/mL) of an ^{111}In-labeled complex via the lateral tail vein under conscious restraint. Approximately 50 µCi sample of each of ^{111}In, and ^{99m}Tc DTPA complexes was also assayed as controls. Rats were placed in metabolism cages immediately after injection and were provided free access to water. Rats that were housed for 24 hours were allowed access to food and water. Three rats per group were euthanized at 15, 60, 120 min and 24 hours post injection. Whole organs and tissue specimens were rinsed with 0.9% sodium chloride Injection U.S.P., blotted dry and weighed. Tissues collected and weighed included: blood, liver, kidney, heart, lungs, muscle and spleen. A portion of the tail where the injection was made was also assayed for radioactivity. Duplicate 0.50 mL samples of heparinized whole blood were pipetted, and two approximately 1 gram samples of liver were weighed for assay of radioactivity. Urine specimens were collected from rats sacrificed at 15, 60 and 120 min. After euthanasia, the cages were rinsed with tap water and the volume of combined urine and rinse was measured. A 0.50 mL aliquot of urine (with rinse) was assayed for radioactivity. After the feces were collected for the 24 hour rats, the urine collection was the same as described above. Tissues, urine and feces were counted by gamma scintillation (Packard Cobra B5005 Gamma Counter).

Injection solution standards were prepared by diluting the injection solutions 1:100 using 0.9% sodium chloride Injection U.S.P. The diluted standards were dispensed in 0.05 mL aliquots with 0.95 ml tap water into tubes for counting. Each sample was assayed for 1 min by gamma scintillation using a gamma counter with the windows set to detect photoelectric energies of each specific isotope assayed (80-500 keV for ^{111}In, 40-240 keV for ^{99m}Tc and 15-240 keV for ^{153}Sm). Raw data were reported in counts per minute (CPM). The total amount of ^{111}In, or ^{99m}Tc administered to each rat was determined by assaying standards prepared from each injection solution. Decay of radioactivity in sample specimens was corrected by averaging the CPM for the standards preceding and immediately following each set of samples.

The percent injected dose (ID) per gram or mL tissue was calculated for those tissues in which tissue weights (or volume) were measured. The percent ID per organ was calculated for all organs. For the purpose of calculation, total blood volume was assumed to represent 5.0% of the initial body weight and muscle mass was to represent 45.5%. For urine and feces, the percent ID in the entire contents was determined. Percent ID for each tissue, fluid and excreta were normalized for the activity remaining at the injection site by subtracting CPM at the injection site from total CPM administered. The percent recovery was calculated for individual animals by adding percent ID values for all organs and excreta. Group means and standard errors were calculated for percent ID per gram and percent ID per organ at each time point using the normalized values. In order to determine whether the complexes are cleared through glomerular filtration or tubular secretion pathway, the rats were first administered a dose of probenecid 35 mg/kg and 70 mg/kg intravenously 10 minutes prior to the test substance.

3. RESULTS AND DISCUSSION

3.1. Fluorescence studies.

The relative fluorescence enhancements of various Eu^{3+} complexes in water compared to Eu-DTPA (**4c**) are shown in Table 1. The samples were excited at 350-400 nm depending on their respective excitation maxima. The emission intensities were recorded at the typical Eu^{3+} emission bands at 594, 615, and 692 nm. All three bands showed the same degree of enhancement, but the intensities at 615 nm were used for the purpose of calculating relative fluorescence.

As would be expected on the basis of cross sectional area, complexes with bicyclic aromatic antennae, viz., **7-13**, exhibited greater enhancement than the corresponding monocyclic derivatives **5** and **6**. Within the three types of bicyclic antennae (naphthyl, coumaryl, and quinoxalinyl), and within the three quinoxaline derivatives **10-13**, there is remarkable

Table 1. Fluorescence Enhancement of Eu^{3+} Emission by Aromatic Antennae

Europium Complex	**Relative Fluorescence**[a]
Eu-DTPA	1
Salicyl-(bis)amide **5**	1[b]
Pyrazinyl-(bis)amide **6**	1
Naphthyl-(mono)amide **7**	10[b]
Carbostyrylyl-(mono)amide **8**	200[c]
Coumaryl-(mono)amide **9a**	1000[b]
Coumaryl-(bis)amide **9b**	1800[b]
Quinoxalinyl-(mono)amide **10**	20
Quinoxalinyl-(mono)amide **11**	20
Quinoxalinyl-(mono)amide ester **12**	1200
Quinoxalinyl-(mono)amide **13a**	2700
Quinoxalinyl-(bis)amide **13b**	1800
α-Napthylurethane **14a**	Quenching of Eu^{3+} fluorescence
β-Napthylurethane **14b**	Quenching of Eu^{3+} fluorescence
EDTA-Oxine **15**	Quenching of Eu^{3+} fluorescence
DTPA-Oxine **16**	Quenching of Eu^{3+} fluorescence

[a] Values rounded to the nearest unit, tens, or hundreds. [b] Ref 15. [c] Ref. 14

difference in fluorescence enhancement property. Coumarin (**9a** and **9b**) and quinoxaline (**13a** and **13b**) derivatives exhibited a large (1000- and 2700-fold respectively) fluorescence enhancement, whereas the carboystyryl (**8**) and quinoxaline derivatives **10** and **11** showing modest or weak enhancement respectively. Substantial difference in fluorescent property between the five quinoxaline derivatives **10-13** is also remarkable, particularly the two-fold difference in enhancement between the ester **12** and the acid **13**. Cursory inspection of the bicyclic antennae in **7**, **8**, **9**, **11**, **12**, and **13** indicates that the carbocyclic ring is electron rich and the heterocyclic ring is electron deficient, whereas in **10**, both electron donation and withdrawal takes place in the heterocyclic pyrazine ring. Secondly, large change in electric dipole takes place in going from the ester to **12** to the carboxylate **13a** (or **13b**), but the precise electronic mechanism governing the enhancement property remains to be investigated. Surprisingly, the bisamide derivative **13b** exhibited about 33% less enhancement compared to the monoamide **13a,** which is the opposite of what was observed between the coumaryl derivatives **9a** and **9b**. Again, surprisingly, in complexes **14a** and **14b**, the characteristic Eu^{3+} emission bands are completely lost and are replaced by a very broad emission band with λ_{max} at about 355 nm. Furthermore, the α-naphthyl compound **14a** is about three times more fluorescent than the β-naphthyl analog **14b**. It appears in this particular case, FRET is taking place from metal to the ligand. Finally, both the EDTA and the DTPA quinoline complexes, **15** and **16** respectively, not only exhibited loss of the characteristic Eu^{3+} emission bands, but these compounds did not exhibit detectable levels of fluorescence, even the in ultraviolet region (< 400 nm).

3.1. Renal clearance studies.

The clearance patterns of ^{99m}Tc-DTPA (**4a)** and ^{111}In-DTPA (**4b**) are shown in Figs. 3 and 4 respectively, and the data is consistent with the previous studies. The quinoxalinyl complex **13c** (i.e. radioactive indium analog of **13a**) exhibited renal clearance similar to that of, akin to that ^{111}In-DTPA (Fig. 5). In order to ascertain whether the complex **13c** clears via glomerular filtration, the rats were first treated with probenecid [25, 26] to block the tubular secretion pathway, and then were administered with **13c**. The results are shown in Table 2. The control compound, ^{99m}Tc-MAG_3 (MAG_3 = mercaptoacetylglycylglylcyglycine)[25] showed substantial decrease in clearance upon probenecid treatment, whereas ^{111}In complex **4b** showed no change in its clearance rate. Although the clearance rate of **13c** is similar to that of **4b** under untreated condition, significantly enhanced clearance rate is observed with probenecid-treated **13c**, and this behavior is also dose-dependent. Such increase in renal clearance rate upon probenecid-treatment has been observed previously with the antitumor agent, cisplatin.[27] In this case, it was suggested that probenecid could competitively inhibit the reabsorption of Pt or Pt species, thus promoting a net secretion.[27] Based on the reported pKa's of the carboxyl groups in DTPA and its derivatives,[20] the pKa's of the carboxyl groups in ligands **7-12** should be below 3. Thus, complex **13c** would be completely

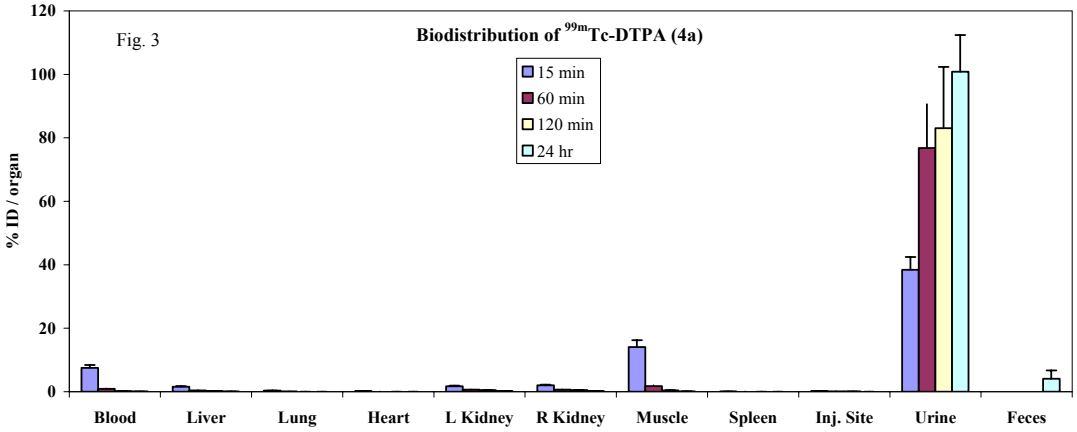


Fig. 3
Biodistribution of 99mTc-DTPA (4a)
15 min
60 min
120 min
24 hr
% ID / organ
120
100
80
60
40
20
0
Blood
Liver
Lung
Heart
L Kidney
R Kidney
Muscle
Spleen
Inj. Site
Urine
Feces

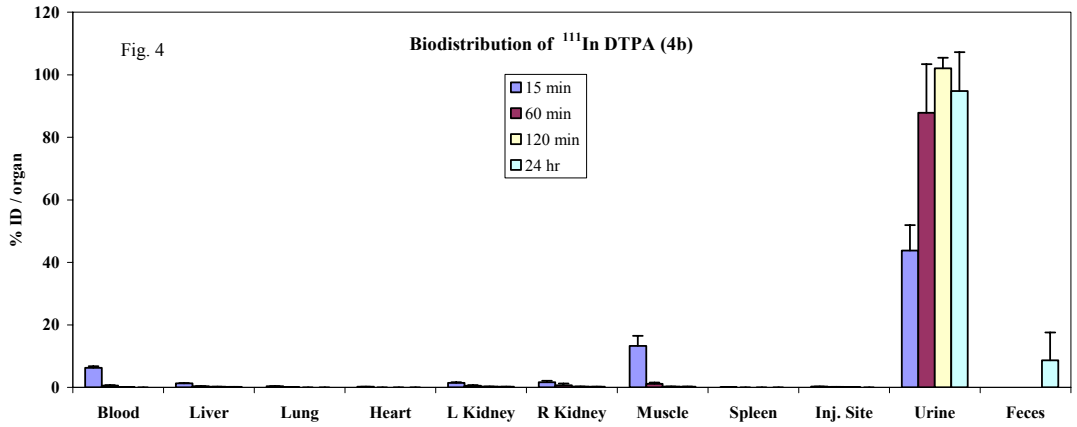


Fig. 4
Biodistribution of 111In DTPA (4b)
15 min
60 min
120 min
24 hr
% ID / organ
120
100
80
60
40
20
0
Blood
Liver
Lung
Heart
L Kidney
R Kidney
Muscle
Spleen
Inj. Site
Urine
Feces

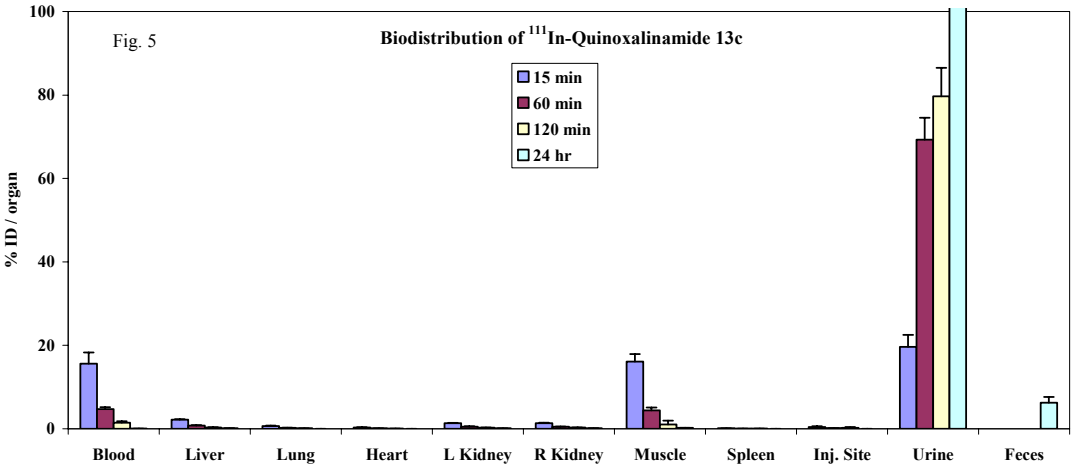


Fig. 5
Biodistribution of 111In-Quinoxalinamide 13c
15 min
60 min
120 min
24 hr
% ID / organ
100
80
60
40
20
0
Blood
Liver
Lung
Heart
L Kidney
R Kidney
Muscle
Spleen
Inj. Site
Urine
Feces

ionized in the kidneys. Since passive tubular reabsorption requires the molecules to be in the undissociated state,[28] complex **13c** would not be expected to undergo tubular reabsorption, but would remain in the lumen and be eliminated in the urine. Furthermore, since probenecid is known to inhibit OAT pathway, complex **13c**, if it is secreted by anion transport mechanism, would be expected to undergo slower clearance, akin to that of complex **5**, but the opposite effect was observed.

Table 2. Effect of Probenecid on the Clearance Rates[a] of Metal Complexes

	Probenecid (mg/kg)		
Complex	0	35	70
^{99m}Tc-MAG$_3$	11.8 ± 2.1	6.4 ± 1.0	4.6 ± 0.6
^{111}In-DTPA (**4b**)	4.3 ± 0.2	4.4 ± 0.7	Not Determined
^{111}In-Complex **18a**	4.5 ± 0.5	8.1 ± 1.4	6.4 ± 0.5

[a] The units are expressed as mL/min/kg.

4. CONCLUSIONS

Large Eu^{3+} fluorescence enhancement via ligand-metal energy transfer mechanism was achieved with the europium complex **13a**. Indeed, complex **13a** exhibits the highest fluorescent enhancement observed thus far in the DTPA-type metal complexes. The large difference in fluorescence enhancement between **10** and **11**, versus **12** and **13** can be qualitatively rationalized by the differences in the nature, the distance, and the relative orientation of electric dipoles between donor antenna and the acceptor metal ion in these complexes, but high level theoretical studies will be needed to elucidate the underlying electronic effects. The weak fluorescence enhancement observed in **11** could be attributed to the longer distance between the donor and acceptor in **11** compared to **13a**. Complex **13c**, which is the indium analog of **13a**, exhibited excellent renal clearance property that mimics the known metal complex-based GFR agents, albeit it clears at a slower rate than **4c**. The fact that **13c** clears at a faster rate after probenecid treatment compared to untreated **13c** suggests that compound is cleared by the kidneys by a complex mechanism that includes glomerular filtration. It is also apparent from this study that any attempt to coordinate the metal directly to the antenna would probably obviate the ligand-metal FRET. Although the excitation maximum for **13a** is at 357 nm, the large fluorescence enhancement would allow this compound to be excited in the visible region to be useful as an exogenous optical renal function monitoring agent.

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