Photochemistry and Photobiology, 2005, 81: 1499–1504

Spectral Properties of Pro-multimodal Imaging Agents Derived from a NIR Dye and a Metal Chelator

Zongren Zhang and Samuel Achilefu*

Department of Radiology, Washington University School of Medicine, St. Louis, MO

Received 8 June 2005; accepted 11 August 2005; published online 24 August 2005 DOI: 10.1562/2005-06-08-RA-568

ABSTRACT

Monomolecular multimodal imaging agents (MOMIAs) are able to provide complementary diagnostic information of a target diseased tissue. We developed a convenient solid-phase approach to construct two pro-MOMIAs (before incorporating radiometal) derived from 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and cypate, a near-infrared (NIR) fluorescent dye analogous to indocyanine green (ICG). The possible interaction between d orbitals of transition metal DOTA complexes or free metals and the p orbitals of cypate chromophore could quench the fluorescence of pro-MOMIAs. However, we did not observe significant changes in the spectral properties of cypate upon conjugation with DOTA and subsequent chelation with metals. The fluorescence intensity of the chelated and nonmetal-chelated PRO-MOMIAs remained fairly the same in dilute 20% aqueous dimethylsulfoxide (DMSO) solution (1×10^{-6} *M*). Significant reduction in the fluorescence intensity of pro-MOMIAs occurred in the presence of a large excess of metal ions (>1 molar ratio for indium and 20-fold for a copper relative to pro-MOMIA). This study suggests the feasibility of using MOMIAs for combined optical and radioisotope imaging.

INTRODUCTION

Because of the limitations of individual diagnostic imaging techniques, accurate diagnosis of diseases often requires a combination of data from two or more imaging methods (1,2). This multimodal approach typically involves the administration of different image-enhancing agents associated with each technique. Colocalization of the images is complicated by differences in the pharmacokinetics and pharmacodynamics of the imaging agents, resulting in differentials in their biodistribution in target tissue. This impediment could be overcome by covalently linking different imaging agents together to produce monomolecular multimodal imaging agents (MOMIAs).

Imaging agents for diverse imaging modalities such as magnetic resonance imaging (MRI), ultrasound and optical have been fused into macromolecules or nanoparticles through covalent or non-covalent interactions (3–5). More recently, smaller molecular MOMIAs (<5kDa) that combine imaging signals for positron emission topography (PET), MRI and optical technologies have been developed (6,7). Key features of the molecular designs involve the use of a metal chelator and a fluorescent dye. In addition to their use for multimodal imaging, the versatility of these molecules makes it possible to further diversify the imaging methods *via* metal selection. For example, optical methods can be combined with MRI or PET by preparing the 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) complex of MOMIAs with Gd or ^{64}Cu ions, respectively.

A typical metal chelator that has been widely studied in PET and MRI imaging is DOTA derivatives, whose dentate arrangement makes it one of the most successful metal-chelating agents for stable metal complexes with a variety of metal ions. Recently, we successfully optimized the synthesis of a stable near-infrared (NIR) fluorescent dye cypate, a reactive analogue of indocyanine green (ICG) (8). Therefore, we anticipate multimodal imaging agents based on DOTA and cypate could play an important role in this strategy. In addition, emitting fluorescence in the NIR region is expected to significantly reduce tissue autofluorescence, thereby making cypate attractive for constructing MOMIAs.

Nuclear imaging methods such as single photon emission computed tomography (SPECT) and PET are widely used in clinical settings for functional imaging of biological processes. To minimize patients' exposure to ionizing radiation, radionuclides with short half-life are preferred for diagnostic purposes. A problem with this strategy is that multiple readministrations of radiopharmaceuticals will be needed to follow the response of target tissue to treatment. Interestingly, a combination of optical and nuclear imaging would represent an important complementary imaging strategy, where the highly sensitive and short-lived radionuclide method is used to localize the diseases especially in deep tissues and the low energy radiation of the fluorescence signal from optical molecular probes is used to continuously monitor the status of disease for prolonged periods.

* To whom correspondence should be addressed: Department of Radiology, Washington University School of Medicine, 4525 Scott Avenue, CB 8225, St. Louis, MO 63110, USA. Fax: 314-747-5191; e-mail: achilefus@mir.wustl.edu

Abbreviations: DCM, dichloromethane; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl; DIC, *N,N'*-diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; ESI, electrospray ionization; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; ICG, indocyanine green; MOMIAs, monomolecular multimodal imaging agents; MRI, magnetic resonance imaging; Mtt, 4-methyltrityl; NIR, near infrared; PET, positron emission topography; TFE, trifluoroethanol; TFA, trifluoroacitic acid; TIS, triisopropylsilane.

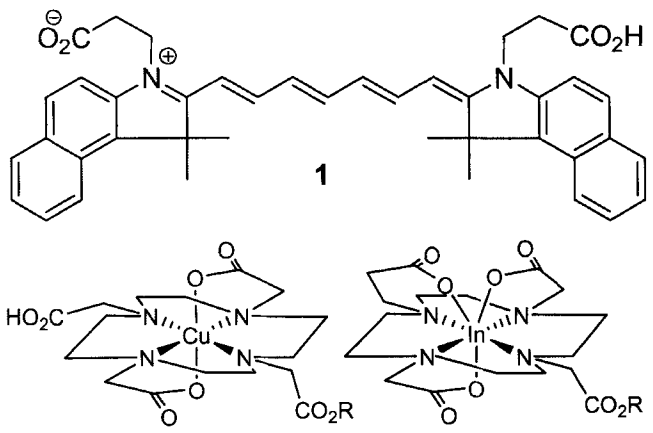


Figure 1. Structure of cypate and DOTA metal complexes, R represents covalently linked cypate.

Logically, metals can quench the fluorescence of cypate through interaction of the metal d orbitals with the p-bonds of the dye. In this study, we assessed the influence of metal ions on the photostability and sensitivity of cypate upon metal complexation to DOTA and in the presence of excess metals. Our results suggest that the pro-MOMIAs† retained their fluorescence emission, except when excess metal ions were added to the solution. Because we expect to use a 1:1 ratio of cypate to radiometal–DOTA complex for radionuclear-optical imaging, our results suggest that fluorescence quenching may not pose a significant hindrance to the use of MOMIAs for coregistration of tissue volume by different imaging methods.

MATERIALS AND METHODS

General. Solvents, reagents and chemicals were purchased from a commercial source (Aldrich, Milwaukee, MI) without further purification unless otherwise stated. In most cases, analytical samples were further purified *via* semipreparative HPLC on a 25 × 2.2 cm Vydac C-18 column (Vydac, Hesperia, CA) equipped with an ultraviolet-visible (UV-vis) monitor. Typically, the resins were washed sequentially with a mixture of dichloromethane/dimethylformamide (DCM/DMF), MeOH/H_2O and DCM/DMF. This washing protocol was used for all solid-phase synthesis unless stated differently in the text. UV-vis studies were made on a Beckman Coulter DU 640 UV-vis spectrometer (Fullerton, CA). Fluorescent emission spectra were recorded on a Jobin Yvon-Spex Fluorolog-3 spectrofluorometer (HORIBA Jobin Yvon, Edison, NJ). All compounds used in the spectral studies were dissolved in 20% aq. dimethylsulfoxide (DMSO). In all cases, the same sample was used for both the absorption and fluorescence studies in quartz cuvettes. There were less than 5 min intervals between the absorption and emission measurements. For metal complexes, NH_4OAc buffer was used to ensure proper pH for the complexation. Mass spectral analysis (MS) was performed on Waters Micromass ZQ4000 spectrometer (Milford, MA) using an electrospray ionization (ESI) method.

Cypate (**1**). A solution of Ac_2O (22.6 μL, 240 μmol) in DCM (100 μL) was added to an ice-cooled solution of glutaconic aldehyde dianilide hydrochloride (0.57 mg, 200 μmol) and diisopropylethylamine (DIEA, 70 μL, 400 μmol) in DCM (0.5 mL). The resulting mixture was stirred for 3 h and added to a refluxing mixture of 1-carboxyethyl-2,3,3-trimethylbenzoindoleninium bromide (200 mg, 550 μmol) prepared by a literature method (9), and NaOAc (78 mg, 950 μmol) in MeCN (1 mL). The mixture was then refluxed for 3 h. The solvent was evaporated, and the residue was washed first with EtOAc and then with 1 *N* HCl to give 0.1 g (80%) of cypate. ^1H NMR (DMSO, 300 MHz): δ 8.24 (d, J = 8.7 Hz, 2H), 7.96 (m, 6H), 7.79 (d, J = 12.9 Hz, 2H), 7.62 (m, 2H), 7.49 (m, 2H), 6.60 (t, J = 12.6 Hz, 1H), 6.46 (d, J = 13.5 Hz, 2H), 4.43 (bt, 4H), 3.77 (t, J = 6.3 Hz, 4H), 1.91 (s, 12H); MS/EI: 625 (M^+).

Tri-t-butyl DOTA-ε-aminohexyl amide (**2**). 1,6-Hexanediamine (**2a,** 372 mg, 3.2 mmol) in DMF (1 mL) was added to a vial containing 2-chlorotrityl resin (1.6 meg/g, 164 mg, 262 μmol) in DCM (4 mL). The mixture was then vibrated (2 h), filtered and the resin was washed as described above. To the resin was then added a solution of tri-*t*-butyl DOTA (**2b,** 300 mg, 524 μmol), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 209 mg, 550 μmol) and *N*-hydroxybenzotriazole (HOBt, 80 mg, 525 μmol) in DMF/DCM (1/1, 10 mL). After mixing the content, DIEA (138 μL, 786 μmol) was added, and the mixture was vibrated for 2 h before washing. Cleavage of **2** was achieved by mixing the resin with a mixture of AcOH (400 μL)/trifluoroethanol (TFE, 800 μL)/DCM (2.8 mL) for 1 h. The mixture was filtered and the resin was further washed with TFE/DCM (2/8, 2 × 4 mL). The filtrate and washings were combined and evaporated under vacuum to give 85 mg (48%) of **2**. The product was used without further purification. MS/ESI: 671 ($M+H^+$).

N-(tri-t-butyl DOTA)-N′-ε-cypate-hexane diamide (**3**). Cypate **1** (80 mg, 127 μmol) in DMF (2 mL)/DCM (2mL) was added to 2-chlorotrityl resin (1.6 meg/g, 40 mg, 64 μmol), preswelled with DCM. After a brief shaking, K_2CO_3 (35 mg, 255 μmol) was added as solid. The resulting mixture was vibrated for 4 h. The mixture was filtered and the resin was washed sequentially with DMF/H_2O/DMF/DCM and the resin was dried under vacuum. After drying, tri-*t*-butyl DOTA-ε-aminohexyl amide **2** (85 mg, 127 μmol) and *N,N′*-diisopropylcarbodiimide (DIC, 108 μL, 700 μmol) in DCM (2 mL) were added, and the mixture was mixed for 5 h. The mixture was filtered, and the resin was washed with DMF/DCM. The product was cleaved as described above for **2** to give 25 mg green solid residue. Pure product was isolated by semipreparative HPLC (40%–70% MeCN in H_2O containing 1% TFA in 100 min). MS/ESI: 1277 (M^+).

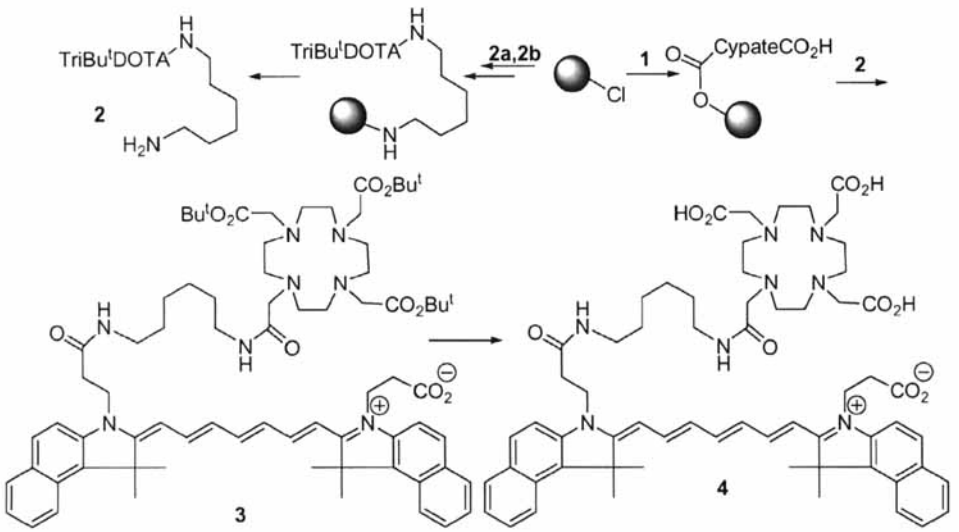
N-DOTA-N′-ε-cypate-hexane diamide (**4**). *N*-(tri-*t*-butyl DOTA)-*N*-ε-cypate-hexane diamide **3** (10.5 mg, 8.2 μmol) in TFA (2 mL) was stirred for 2 h at room temperature. TFA was removed, and the resulting green solid was washed with ether and the compound was isolated by semipreparative HPLC (40%–70% MeCN in H_2O containing 1% TFA in 100 min) to give 1.3 mg of **4** as green solid. MS/ESI: 1109 (M^+).

N-α-cypate-N-ε-DOTA-lysine diamide (**5**). DIC (8 μL, 50 μmol) was added to a sealed vial containing Fmoc-Lys(Mtt)-OH (65 mg, 100 μmol) in DMF (15 μL)/DCM (400 μL), precooled on an ice bath. The mixture was stirred for 20 min at 0°C to give white precipitate. The solvent was removed *via* a rotary evaporator, and the residue was transferred into a vial containing Wang resin (0.82 mmol/g, 42 mg, 35 μmol), preswelled in DMF. 4-Dimethylaminopyridine (DMAP, 0.42 mg, 3.5 μmol) was added, and the resin mixture was vibrated for 4 h. The mixture was filtered, and the resin was washed as described section above. The lysine-bound resin was then treated (3 × 2 min) with a solution of DCM/TFA/triisopropylsilane (TIS; 94:1:5; 3 × 500 μL) and washed to remove 4-methyltrityl (Mtt). A mixture of DMF (300 μL)/DCM (300 μL) was added, followed by the addition of tri *t*-butyl DOTA (56 mg, 98 μmol), HOBt (15 mg, 98 μmol) and HBTU (37 mg, 98 μmol). After mixing the content briefly, DIEA (34 μL, 197 μmol) was added, and the resin mixture was vibrated for 20 h. The mixture was filtered and washed before and after removal of Fmoc with piperidine in DMF (20%, 2 × 8 mL, 20 min). Cypate **1** (164 μmol, 100 mg), HBTU (164 μmol, 62 mg), HOBt (164 μmol, 25 mg), DMF (2 mL)/DCM (2 mL) and DIEA (38 μL, 220 μmol) were added sequentially to the resin. The mixture was then vibrated (5 h), filtered and washed. The crude product was cleaved from the resin by shaking the resin in TFA (2 mL) for 3 h. It was then purified by fractional semipreparative HPLC (40%–70% MeCN in H_2O containing 1% TFA in 100 min) to give 15 mg of the compound as green solid. MS/ESI: 1139 (M^+).

N-DOTA-Cu(II)-N′-ε-cypate-hexane diamide (**6**). Solutions of **4** (1.34 mg, 1.21 μmol) in MeCN (0.2 mL), $Cu(OAc)_2$ in H_2O (13.6 m*M*, 105 μL) and NH_4OAc (0.1 *M*, 3 mL) were mixed together and stirred for 1 h at 50°C. The solvent was removed, and the residue was washed with H_2O. The resulting solid was suspended in H_2O, and drops of MeCN were added until a clear solution was obtained. The solution was loaded on a silica gel C-18 cartridge. The complex on cartridge was further washed with H_2O and removed from the cartridge with a minimum amount of aqueous MeCN. The eluent was lyophilized to give 0.3 mg of the copper complex as a green solid. MS/ESI: 1170 (M^+).

N-α-cypate-N-ε-DOTA-Cu(II)-lysine diamide (**7**). $Cu(OAc)_2$ (9.1 mg, 50 μmol) and NH_4OAc (771 mg, 10 mmol) were dissolved in H_2O (9.0 mL)

† MOMIAs contain two signaling antennas for multimodal imaging. We are referring to the compounds used in this particular study as pro-MOMIAs because we did not use the radioactive metal chelates. Note that the second antenna could be radioisotope or other moieties such as paramagnetic metals.



Scheme 1. Synthesis of *N*-DOTA-*N'*-ε-cypate-hexane diamide (**4**).

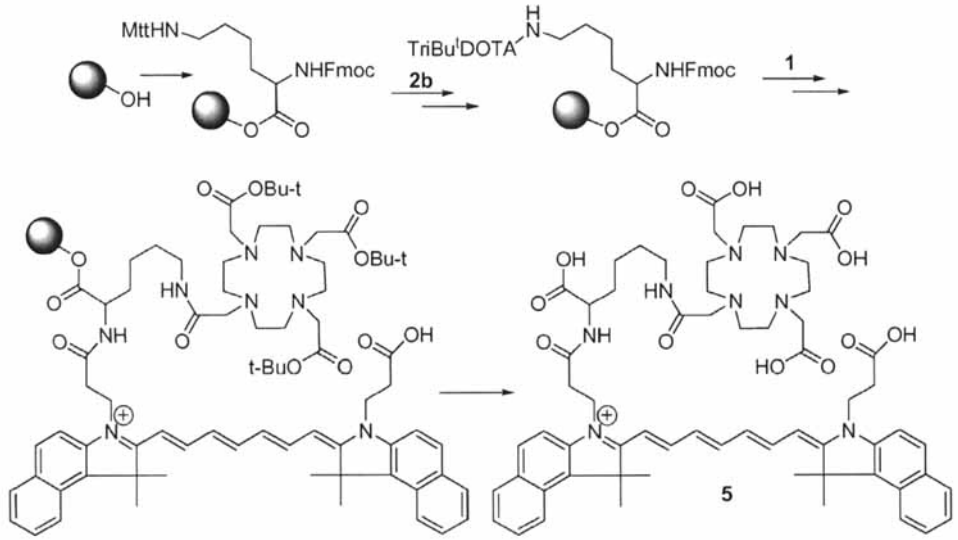
and MeCN (1.0 mL) to give a solution of 1.0 m*M* Cu(II)/0.2 M NH_4OAc. Compound **5** (10.0 mg, 8.78 μmol) was added to 2 mL of the above solution. The solution was stirred at 50°C and monitored by thin-layer chromatography (TLC, 3:1 MeCN/H_2O) until the green spot became static. The mixture was lyophilized and washed with Et_2O (10 mL), Et_2O/MeCN (3 × 10 mL) and MeCN (2 × 5 mL). The residue was dried before a final wash with H_2O (3 × 10 mL). The resulting solid was dried in a vacuum to give 5.6 mg of the copper complex as a green solid. MS/EI: 1200 (M^+).

N-DOTA-In(III)-N'-ε-cypate-hexane diamide (**8**). $InCl_3$ (18.5 mg, 16 μmol) was mixed with a solution of compound **4** (15 mg, 13 μmol) in MeCN (500 μL)/H_2O (500 μL). The product was purified by semipreparative HPLC (40%–70% MeCN in H_2O containing 1% TFA in 100 min) to obtain 2 mg of **8** as bluish green solid after lyophilizing. MS/EI: 1221 (M^+).

Spectral measurements. For absorption and emission measurements, compounds **3**–**8** were carefully weighed on a precision balance and first dissolved in a specific volume of DMSO. They were then diluted with a predetermined volume of H_2O to give a solution of 20% aq. DMSO. The freshly prepared solutions were further diluted with 20% aq. DMSO to give dilute solutions whose absorbance (A) was controlled at ~0.05 to avoid sample aggregation that occurs at high concentrations. The solutions were used to measure absorption and emission spectra of the compounds sequentially. To assess the influence of complexation on the spectral properties of the compounds, dilute solutions of **4** or **5** of known concentrations and volume were prepared. Increasing concentrations of metal salts were added to the solution, along with or without pH buffer. The resulting mixtures were stirred or warmed, based on the conditions used in their synthesis. UV and fluorescence measurements were conducted before and after each addition of metal solution. Adequate controls to maintain identical conditions for each measurement were used such that the only variable was the metal concentration. For example, **4** (0.78 mg, 0.70 μmol) was dissolved in DMSO (2.00 mL) and diluted with H_2O (8.00 mL) to give concentrated solution (10.00 mL, 7.03×10^{-5} M) in 20% aq DMSO. A given volume (100 μL) of the solution was further diluted with 3.90 mL of 20% aq DMSO to give 1.75×10^{-6} *M* solution. This final solution was used for the spectral study. To evaluate the effect of metal complexation on the spectral properties of the pro-MOMIA, 50 μL of NH_4OH solution (3.6×10^{-3} *M*) was added to 4.00 mL of the dilute solution of **4**, followed by sequential addition of different volumes (0, 10, 30, 60 and 100 μL) of Cu(II) solution (7.27×10^{-4} *M*) prepared from $CuCl_2 \cdot 2H_2O$ in 20% aq. DMSO. After each addition of the metal solution, the resulting solution was swirled with pipette tip and warmed for 1 h at 50°C before recording the absorption and emission spectra. Dilution effect resulting from the addition of metal and buffer solutions was corrected in data treatment.

RESULTS AND DISCUSSION

Structurally analogous and photochemically similar to ICG, cypate (Fig. 1) has two carboxyl groups. which make it readily reactive with amines for preparing peptide-based optical imaging agents (9). With proper protection, cypate could also be conjugated to



Scheme 2. Synthesis of *N*-α-cypate-*N*-ε-DOTA-lysine diamide (**5**).

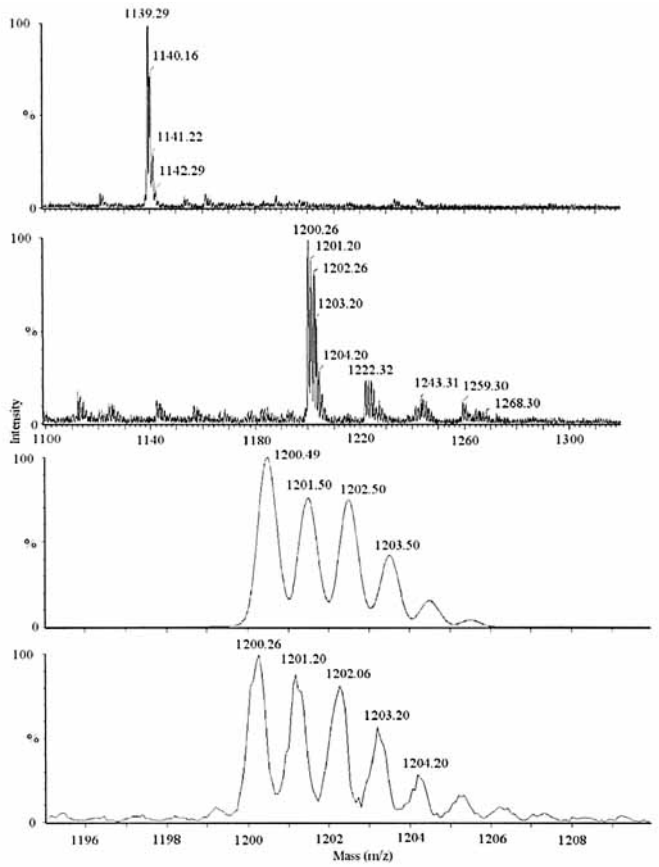


Figure 2. Comparative mass spectra of **5**, its Cu(II) complex **7**, theoretical and actual isotope pattern of **7** (from top to bottom).

a DOTA molecule in a similar fashion *via* a diamine linker. The resulting molecule should be able to incorporate a metal ion into the DOTA unit to give multimodal imaging agents. DOTA chelates of radioactive copper (^{64}Cu) and indium (^{111}In) are widely used in PET and SPECT studies. Therefore, their nonradioactive isotopes are excellent models for assessing metal effects on the spectral properties of pro-MOMIAs. Taking Cu(II) and In(III) as examples, the incorporation of metal ion will give a coordination number of 6 or 7 depending the oxidation state of the metal ions (Fig. 1).

DOTA-protected multimodal agent **3** (Scheme 1) has been synthesized by a solution method through careful monitoring of the reaction conditions (10). Though successful, repeatability was poor because slight alterations of the ratio of starting materials and reagents could exclusively lead to byproducts. The multiple functionality features of the building blocks led us to explore its synthesis on solid support as outlined in Scheme 1. Cypate was loaded on a trityl chloride resin and subsequently reacted with **2** and also synthesized on trityl chloride resin. Cleavage under mild acidic conditions gave **3** without removing the DOTA-protecting group. The structural feature of the protected multimodal agent **3** facilitates further modification of the compound *via* the other free carboxyl group. For example, a receptor-specific MOMIA can be obtained by reaction of the carboxyl group with peptides. Additionally, the excretion pathway or biodistribution of the MOMIAs can be modified by conjugating a variety of hydrophilic or lipophilic groups to the pendant carboxyl group. Alternatively, **3** can be converted readily into **4** to release the chelating power of the

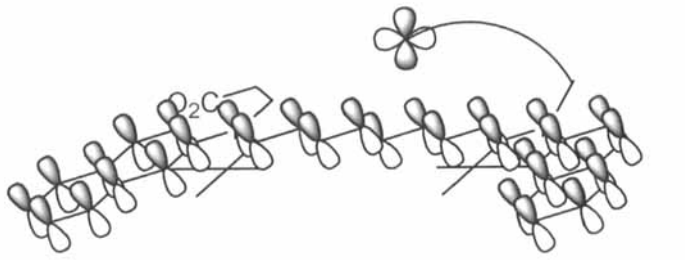


Figure 3. Molecular orbital representation of pro-MOMIAs.

DOTA unit. Thus **3** is a versatile intermediate for the preparation of a variety of MOMIAs.

We also developed a solid-phase approach for preparing the multimodal agent **5** (Scheme 2). Initially we used the orthogonally protected Fmoc-Lys(Dde)-OH amino acid to couple tri-*t*-butyl DOTA after selective removal of Fmoc group. Although **5** was obtained, both Fmoc and 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) are unstable under basic conditions and may result in inadvertent removal of both protecting groups. Consequently, we chose Fmoc-Lys(Mtt)-OH for the synthesis of **5** because both protecting groups are removed under very diverse conditions and resulted in higher yield of **5**.

Combination of cypate with DOTA into one molecule (**4**, **5**) did not affect the chelating capability of DOTA. We chose nonradioactive Cu(II) and In(III) instead of the radioactive isotopes in the study of metal complexation and optical activity for safety reasons. They were incorporated into DOTA units following procedures developed for radiolabeling the chelator (11–13). In(III) complex was found to be stable and readily formed even under mild acidic conditions. Incorporation of Cu(II) required the use of pH buffer and warm incubation conditions. Complexation of the metal can be monitored by TLC using aq. MeCN solvent system because the pattern, shape and position (R_f) of the pro-MOMIAs are visually observable. In contrast to metal-free multimodal agents **4** and **5**, the metal complexes were found to be stationary at the TLC plate base after complexation. The In(III) complex can be separated by HPLC in the presence of 1% TFA using typical elution protocol for peptide separation (40%–70% MeCN in H_2O containing 1% TFA in 100 min). The Cu(II) complex decomposed under this condition, as evidenced by MS analysis. The Cu(II) complex was eventually isolated using C-18 cartridge. Shown in Fig. 2 is a typical mass spectral study of the synthesis, separation and isolation of pro-MOMIAs and their metal complexes.

Optical activity (color) of transition metal ions such as Cu(II) arise from d orbitals splitting in ligand field (14). Though the dye and DOTA-metal complexes are covalently bound, d orbitals of DOTA metal complex and p orbitals of cypate chromophore are isolated. However, there exists the possibility of p–d orbital interaction through space (Fig. 3). This interaction could enhance, reduce or even quench the fluorescence of cypate. The later scenario would be detrimental to the use of MOMIAs for fluorescence imaging studies. Over space interaction relies on close contact and proper orientation of the two units (15). It is thus interesting to compare and evaluate photochemical properties of the multimodal agents.

Essentially aromatic in character, indocyanine dyes such as cypate are known to aggregate in aqueous media at high concentrations. Significant aggregation could distort absorption and thus affects the accuracy of spectral measurements. For this reason, absorption and fluorescence characterization of the

Table 1. Absorption and emission of **1**, **4–8** in 20% aq. DMSO

Compounds	1	4	5	6	7	8
C (10^{-6} *M*)	0.25	1.75	0.58		0.95	
Molar abs (ε 10^5 M^{-1} cm^{-1})	1.58	1.63	1.67		1.23	
Abs λ_{max} (nm)	782	784	781	784	784	784
Em λ_{max} (nm)	810	806	805	802	803	804

multimodal agents were conducted in dilute solutions. Shown in Table 1 are the absorption and emission properties of cypate **1**, multimodal agents **4**, **5**, their Cu(II) complexes **6**, **7** and In(III) complex **8**, taken in 20% aq. DMSO. On one hand, no major peak shift was observed in the absorption spectra upon DOTA conjugation and metal incorporation. On the other hand, the peak shift was noticeable in the emission spectra, which was excited 20 nm below the maximum absorption to obtain the full emission spectra. The homologous structural features of **1**, **4**, **6** and **8** make them interesting for comparative study. Their spectra are represented in Fig. 4, where indium complexation led to relatively larger change in the absorption spectra. The study demonstrates that DOTA conjugation and metal complexation have little effect on the spectral stability of cypate, a critical criterion in developing optical/PET multimodal imaging agents.

We also quantified the influence of metal complexation on the photostability of the pro-MOMIAs. Using isolated multimodal agents, enhancement of absorption and emission properties were observed in certain cases in a previous study (10). To further explore this finding, we prepared dilute solutions of **4** at well-controlled volume, pH and concentrations. We measured its absorption and emission in a preset instrumental condition after sequential addition of specific amounts of metal solutions and incubating the mixture for 30 min at 50°C. Figures 5 and 6, showing the influence of Cu(II) and In(III) on the spectral stability of multimodal agent **4**, were obtained after adjusting the final concentration of the pro-MOMIA solution following the addition of the metal solution. The excitation wavelength used in the measurement was fixed 20 nm below maximum absorption of **4** to obtain the full emission profile of the compound. The data suggest that adverse influence of metal ion on the photoactivity of the chromophore is unlikely. This is particularly true for the absorption and emission of Cu(II) and for the absorption of In(III). Although In(III) has a relatively large reduction in the emission intensity at metal:pro-MOMIA ratios >1, its emission

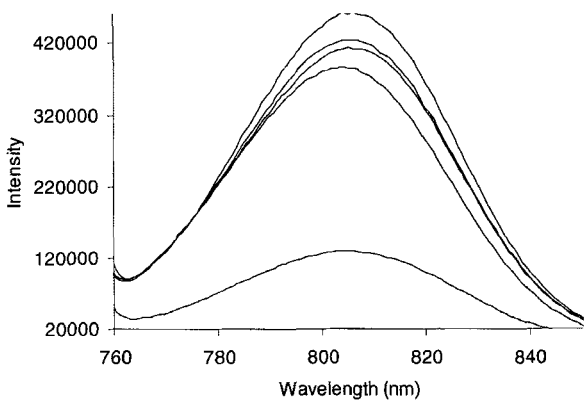


Figure 5. Effects of Cu(II) on the emission of **4** under DOTA complexation conditions. Concentration of **4** is 1.75 × 10^{-6} *M* in 20% aq. DMSO with dilution effect corrected. The ratios of Cu(II) relative to **4** (from top to bottom curve) are 0, 10, 4, 1 and 20.

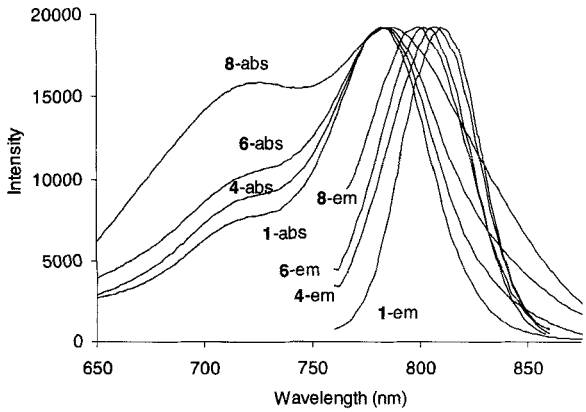


Figure 4. Normalized absorption and emission of **1**, **4**, **6** and **8**. Dilute solutions (~1 × 10^{-6} *M*) in 20% aq. DMSO were used. X = wavelength (nm), Y = absorption/emission intensities after normalization (AU).

signal was strong and detectable at various metal concentrations. At higher concentrations of metal ions (>2 molar ratios relative to pro-MOMIA), the interactions of the metal with the dye may no longer be mediated by the DOTA complex alone but may also involve direct interaction of free metals with the p-bonds of the dye chromophore. Although we do not envisage using excess radioactive materials in the preparation of radioactive MOMIAs for *in vivo* imaging, the chelate may become unstable *in vivo*, thereby allowing the metal to interact directly with the p orbitals of cypate. The findings of this study show that the fluorescence emission of the MOMIA will not be quenched by such an occurrence. Thus differences in the distribution of optical and radionuclear signals within a tissue volume may indicate *in vivo* instability of the MOMIA. Note also that the influence of metal complexation on spectral properties is dependent on the medium. We conducted the present study in 20% aq. DMSO solution because all the pro-MOMIAs are soluble in this solvent mixture, which is currently used in small animal imaging studies (16).

In conclusion, we developed a method to prepare pro-MOMIAs on solid support and studied their spectral properties. The synthetic method is amenable to further derivatization of the pro-MOMIAs to produce target-specific compounds. We did not observe a significant change in the spectral properties of the fluorescent moiety of cypate-based pro-MOMIAs before and after metal chelation. At higher concentrations of the ions (>1 molar ratio for

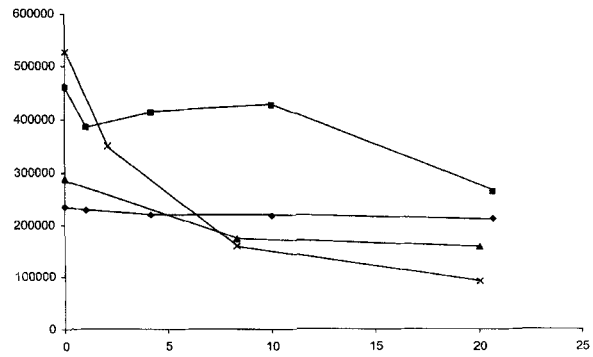


Figure 6. Comparison of the effects of Cu(II) and In(III) on the absorption and emission of **4** under DOTA complexation conditions. X-axis, ratio of metal ion relative to **4** (1.75 × 10^{-6} *M*) in 20% aq. DMSO. Y-axis, absorbance (multiplied 1 × 10^6)/emission (CPS) intensity at maximum wavelength. ■ = Cu(II) on emission, × = In(III) on emission, ▲ = In(III) on absorption, ◆ = Cu(II) on absorption.

indium and 20-fold for copper relative to pro-MOMIA), a decrease in the fluorescence intensity was observed, but the fluorescence emission was detectable at all concentrations studied. This observation suggests that the stability of the metal complexes can be monitored *in vivo*, which will be manifested by detection of the radionuclear and fluorescence signals at different tissue compartments. The findings of this study support the hypothesis that labeling cypate–DOTA conjugates with radiometals can furnish diagnostic information obtained by coregistering optical and radionuclear signals emanating from the same molecule. Ongoing studies to test this hypothesis will be reported elsewhere.

Acknowledgements—This study was supported by the National Institutes of Health (R33 CA100972). We thank Ms. Kexian Liang for technical assistance.

REFERENCES

1. Benaron, D. A. (2002) The future of cancer imaging. *Cancer Metastasis Rev.* **21**, 45–78.
2. Glasspool, R. M. and T. R. J. Evans (2000) Clinical imaging of cancer metastasis. *Eur. J. Cancer* **36**, 1661–1670.
3. Kelly, K. A., J. R. Allport, A. Tsourkas, V. R. Shinde-Patil, L. Josephson and R. Weissleder (2005) Detection of vascular adhesion molecule-1 expression using a novel multimodal nanoparticle. *Circ. Res.* **96**, 327–336.
4. Morawski, A. M., G. A. Lanza and S. A. Wickline (2005) Targeted contrast agents for magnetic resonance imaging and ultrasound. *Curr. Opin. Biotechnol.* **16**, 89–92.
5. Schellenberger, E. A., D. Sosnovik, R. Weissleder and L. Josephson (2004) Magneto/optical annexin V, a multimodal protein. *Bioconjug. Chem.* **15**, 1062–1067.
6. Manning, H. C., T. Goebel, R. C. Thompson, R. R. Price, H. Lee and D. J. Bornhop (2004) Targeted molecular imaging agents for cellular-scale bimodal imaging. *Bioconjug. Chem.* **15**, 1488–1495.
7. Moore, A., Z. Medarova, A. Potthast and G. P. Dai (2004) In vivo targeting of underglycosylated muc-1 tumor antigen using a multimodal imaging probe. *Cancer Res.* **64**, 1821–1827.
8. Zhang, Z. and S. Achilefu (2004) Synthesis and evaluation of polyhydroxylated near-infrared carbocyanine molecular probes. *Org. Lett.* **6**, 2067–2070.
9. Achilefu, S., A. Srinivasan, M. A. Schmidt, H. N. Jimenez, J. E. Bugaj and J. L. Erion (2003) Novel bioactive and stable neurotensin peptide analogues capable of delivering radiopharmaceuticals and molecular beacons to tumors. *J. Med. Chem.* **46**, 3403–3411.
10. Zhang, Z. R., K. Liang, S. Bloch, M. Berezin and S. Achilefu (2005) Monomolecular multimodal fluorescence-radioisotope imaging agents. *Bioconjugate Chem.* **16**, 1232–1239.
11. Li, W. P., J. S. Lewis, J. Kim, J. E. Bugaj, M. A. Johnson, J. L. Erion and C. J. Anderson (2002) DOTA-D-Tyr^1-octreotate: a somatostatin analogue for labeling with metal and halogen radionuclides for cancer imaging and therapy. *Bioconjug. Chem.* **13**, 721–728.
12. Lewis, J. S., R. Laforest, M. R. Lewis and C. J. Anderson (2000) Comparative dosimetry of copper-64 and yttrium-90-labeled somatostatin analogues in a tumor-bearing rat model. *Cancer Biother. Radiopharm.* **15**, 593–604.
13. Anderson, C. J., T. S. Pajeau, W. B. Edwards, E. L. C. Sherman, B. E. Rogers and M. J. Welch (1995) In vitro and in vivo evaluation of copper-64-labeled octreotide conjugates. *J. Nucl. Med.* **36**, 2315–2325.
14. Cotton, F. A (1990) The ligand field theory. In *Chemical Applications of Group Theory*, 3rd ed. pp. 287–288. Wiley, New York.
15. Tachibana, Y., N. Kihara and T. Takata (2004) Asymmetric benzoin condensation catalyzed by chiral rotaxanes tethering a thiazolium salt moiety via the cooperation of the component: can rotaxane be an effective reaction field? *J. Am. Chem. Soc.* **126**, 3438–3439.
16. Achilefu, S., R. B. Dorshow, J. E. Bugaj and R. Rajagopalan (2000) Novel receptor-targeted fluorescent contrast agents for in vivo tumor imaging. *Investig. Radiol.* **35**, 479–485.