Modular Synthesis of DOTA-Metal-Based PSMA-Targeted Imaging Agents for MRI and PET of Prostate Cancer**

Abstract: A practical, convergent synthesis of prostate-specific membrane antigen (PSMA) targeted imaging agents for MRI, PET, and SPECT of prostate cancer has been developed. In this approach, metals chelated to 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) were placed on the side chains of lysine early in the synthesis to form imaging modules. These are coupled to targeting modules, in this case consisting of the PSMA-binding urea DCL, bonded to an activated linker. The modular approach to targeted molecular imaging agents (TMIs) offers distinct advantages. By chelating the MRI contrast metal Gd early, it doubles as a protecting group for DOTA. Standard coupling and deprotection steps may be utilized to assemble the modules into peptides, and the need for tri-tert-butyl protection of DOTA requiring removal by strong acid is averted. This enables mild conjugation of the imaging module to a wide variety of targeting agents in the final step. It was further discovered that two labile metals, La^{3+} or Ce^{3+}, can be used as placeholders in DOTA during the synthesis, then transmetalated in mild acid by Cu^{2+}, Ga^{3+}, In^{3+}, and Y^{3+}, metals used in PET/SPECT. This enables the efficient synthesis of nonradioactive analogues of targeted molecular imaging agents that may be transported or stored until needed. A simple and mild two-step transmetalation, involving de-metalation in dilute acid, followed by rapid chelation of the radioactive metal, may be conveniently performed later at the clinic to provide the TMIs for PET or SPECT.

The clinical limitations of nontargeted molecular imaging agents for prostate cancer (PCa) has led to an exponential increase of investigational targeted imaging agents for magnetic resonance imaging (MRI), positron emission tomography (PET), and single-photon emission computed tomography (SPECT) in both preclinical and clinical research over the past five years.[1,2] In particular, the use of targeted PET agents was found to be remarkably effective in the detection, staging, and active surveillance of PCa.[3–8] Targeted agents for MRI, as well as dual modal agents for SPECT/Optical Imaging, have also been shown to be effective in pre-clinical imaging of active PCa.[9–13] Recently, a study using simultaneous PET/MRI has reported remarkably high accuracy (80–97.5%) in the detection of multiple stages of PCa, underscoring the need for reliable synthetic methods for targeted probes for both PET and MRI of PCa.[14]

The success of targeted molecular imaging agents (TMIs) for PCa has largely been made possible by the discovery of prostate-specific membrane antigen (PSMA) overexpression in PCa cells.[15–17] PSMA, also known as glutamate carboxypeptidase II (GCPII), NAALADase, and folate hydrolase FOLH1, is a well-characterized trans-membrane glycoprotein.[18] Its expression is elevated with increasing PCa stage and grade making it an excellent biomarker for staging, metastasis detection, and image-guided interventions.[19,6] PSMA is also expressed on the neovascularature of other types of carcinomas such as breast and thyroid, making it an important biomarker in the larger scope of cancer research as well.[18,19]

Elucidation of the structure of the PSMA binding site using protein crystallography led to the development of small molecule inhibitors which included amide, phosphonate, and urea substrate analogues.[20,21] Among the most effective of these was a small molecule synthesized by a peptidomimetic urea linkage of lysine to glutamic acid in place of the naturally occurring peptide amide substrate. This urea, designated by the names N-(N-{[(S)-1,3-dicarboxypropyl]-carbamoyl}-S)-L-lysine, DCL, and Glu-Urea-Lys, has emerged as the most effective PSMA inhibitor.[20,21]

Currently, the PCa TMIs most widely investigated are 68Ga-labeled DCL probes for PET imaging, with the agent 68Ga-PSMA-11 (also known as 68Ga-DKFZ-PSMA-11) being most prevalent in clinical studies.[3,7,14] While 68Ga-DKFZ-PSMA-11 employs an acyclic metal-chelating group, HBED-CC, the alternative and well-known cyclic chelator, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), is at the core of many other reported TMIs for both PET and MRI of PCa.[17,22–24] DOTA is also the chelating moiety in 177Lu-PSMA-617, an analogous radiotherapeutic agent in current clinical use for PCa therapy.[25]

The cyclic structure of DOTA allows it to bind strongly to a wide variety of metals. In studies measuring kinetic and thermodynamic stability of chelated lanthanide metals it has been shown that DOTA is superior in complexing gadolinium (Gd^{3+}) with minimal displacement of the metal in blood or other tissues compared to open chain chelating agents such as diethylentriaminepentaacetic acid (DTPA).[26,27]

The stability of Gd within the cyclic DOTA chelating agents versus the weaker binding acyclic chelating agents such as DPTA agents is particularly advantageous in MRI. In contrast to the relatively safe cyclic complexes such as the DOTA-containing Dotarem®, open-chain analogues, such as Magnevist® can lead to gadolinium toxicity due to nephrogenic systemic fibrosis (NSF).[28]

When Gd-DOTA complexes are coupled to small molecules or small peptides, they maintain excellent stability, water solubility, and bioavailability.[24] For these reasons, DOTA continues to be widely utilized as the chelator of choice in MRI contrast agents.

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[**] PSMA = prostate-specific membrane antigen; DOTA = 1,4,7,10-tetraaza
clododecane-1,4,7,10-tetraacetic acid.

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Targeted PET imaging agents provide high sensitivity and functional information and may be fused with images from MRI or CT to provide higher spatial resolution. While not as sensitive as PET, MRI is described as exquisite in precisely depicting the internal prostatic anatomy, margins, tumor extent, and surrounding tissues. \[23\] Additionally, in preoperative reviews, the advantages of high resolution MRI allow surgeons to significantly improve decisions regarding the preservation or resection of neurovascular bundles while performing radical prostatectomies. \[24\]

Utilizing the targeting agent DCL, a series of small molecules containing Gd-DOTA targeted to PSMA was synthesized and found to be remarkably effective for the MRI of PCa in mouse models. \[10\] Apart from this study, there have been few reports of contrast agents for MRI which rely on DCL or other PSMA inhibitors as targeting moieties.

As part of our overall research goals, we developed a modular, peptide-based synthesis of TMIA in which imaging modules, comprised of dyes or chelated metals bonded to the side chain of lysines, may be conjugated to a wide variety of targeting agents. \[29, 30\] Given the prominence of DCL-based, PSMA-targeted agents for PET/SPECT and MRI in pre-clinical and clinical research, we felt it was important to explore this method as an alternative means of assembling metal-based PSMA-targeted imaging agents, for both modalities. This example would also demonstrate the wider utility of the modular method in molecular imaging research.

In the existing syntheses of metal-based targeted agents for PET, SPECT, and MRI, the metal is routinely introduced in the last step. \[31–34\] This generally requires incorporation of an activated linker form of DOTA near the end of the synthesis or the incorporation of a tri-tert-butyl ester protection scheme on DOTA, which further requires the use of relatively harsh tri-fluoroacetic acid (TFA) in the final steps. These conditions may preclude the use of desirable functional groups and may adversely restrict the order of synthesis. If harsh TFA is required in the final steps it could degrade many targeting peptides, proteins, or other delicate small targeting molecules.

In the approach presented here, metals chelated to DOTA are placed on the side chains of amino acids early in the synthesis to form imaging modules. We verified an earlier report that by chelating the metal early, it can double as a protecting group for DOTA, averting the need for the tri-tert-butyl protection. \[25\] The imaging module may thus be incorporated into peptides using standard peptide synthesis conditions, or coupled to linkers and conjugated to targeting agents.

With the ability to conjugate the targeting group in the last step under mild conditions, and with no further treatment of strong acids or bases, a variety of targeting systems including sensitive small molecules, peptides, and proteins and antibodies may be coupled to a given modular imaging system in the final steps of the synthesis.

The optimized, one-step synthesis of the imaging modules containing chelating metals is shown in Scheme 1. In this approach, the starting Fmoc-Lys-NH$_2$ (1a) was prepared by amidation of the commercially available Fmoc-Lys(Boc)-OH (Fmoc = fluorenlymethoxycarbonyl; Boc = tert-butoxycarbonyl), followed by removal of the Boc group. The acid form, Fmoc-dLys-OH (1b), available commercially, is useful for creating imaging modules that may be assimilated into peptides by using standard coupling and Fmoc deprotection methodology. The use of dLys stems from related work in which a second imaging module is added to form dual modal agents and is used to impart an increase in proteolytic stability. \[26\]

This method can be applied to the synthesis of gadolinium modules for MRI directly (M$_1$ = Gd, Scheme 1), with no further transmetalation necessary. To facilitate the synthesis of radio labeled agents for PET and SPECT, we made use of literature reports that two metals, lanthanum and cerium (M$_1$ = La$^{3+}$ or Ce$^{3+}$), are significantly more labile than Gd, and determined that these chelates are stable to conditions of standard peptide syntheses. \[35\] The two metals can thus be used as placeholders, then transmetalated by Cu$^{2+}$, Ga$^{3+}$, In$^{3+}$, and Y$^{3+}$, chosen as nonradioactive models of the clinically relevant PET species.

In this synthesis of TMIA for PSMA, we utilized imaging modules containing La$^{3+}$ as a placeholder (2c and 3b in Scheme 3) as it is displaced more rapidly than Ce$^{3+}$, but we showed earlier that Ce$^{3+}$ may be used in cases in which a more stable placeholder may be preferable.

Scheme 1. Synthesis of imaging modules: 1a: R = NH$_2$; 1b: R = OH; 2a: R = NH$_2$, M$_1$ = Gd (for MRI); 1b, 2b: R = OH, M$_1$ = Gd; 2c: R = NH$_2$, M$_1$ = La (placeholder for PET); 3a: M$_1$ = Gd, 3b: M = La (placeholder for PET).
To optimize the transmetalation step for the TMIAs, we modified methods by Sherry and others\cite{36, 37} used for measuring kinetics of acid-promoted displacement of La$^{3+}$ and Ce$^{3+}$ from their DOTA chelates using the modules, with the goal of providing the mildest conditions possible while completely removing the placeholder. While the transmetalation can be carried out with the metal present, long reaction times are required which are not conducive for radiolabels. After verifying that the rate-limiting step in mild acid was the de-metalation, we solved this by developing a two-step method of stirring the placeholder module or TMIA in dilute acid (0.2 M TFA) first, for 16–24 hours to de-metalate, followed by adding the PET metal which chelates to the DOTA rapidly.

To confirm complete removal of the placeholder metal, each step and all final products were monitored to the detection limit of the La$^{3+}$ species by LCMS. Using this method we found that Cu$^{2+}$ and Y$^{3+}$ chelation was complete, but the La$^{3+}$ competed with Ga$^{3+}$ and In$^{3+}$ for remetalation under the above conditions after neutralization. To ensure complete transmetalation in those cases, a method was devised to adhere the de-metalated TMIA in mild acid solution to a C-18 SPE cartridge or HPLC column, washing away the La$^{3+}$, then treating the column with a 5 mM solution of Ga$^{3+}$ or In$^{3+}$, and then eluting the pure product with an acetonitrile/water gradient.

To synthesize the targeting group DCL, two main methods are reported for preparation, with various modifications in the majority of subsequent studies.\cite{20, 21} These methods paved the way for a number of clinical trials including PSMA-targeted SPECT/CT and positron emission tomography (PET) using $^{18}$F-labelled PSMA urea inhibitor and a targeted agent for endoradiotherapy of PCa\cite{21, 22, 34, 38, 39}.

Our route to DCL is a modification of the synthesis reported by the Pomper group. It begins with the orthogonally protected glutamic acid, Boc-Glu(PMB)-OPMB, available from Boc-Glu-OH, with selective deprotection in acetonitrile to yield the deprotected amine, H-Glu(PMB)-OPMB (4) in high yield. The triply differentially protected lysine, Fmoc-Lys(Boc)-OPMB prepared from the readily available Fmoc-Lys(Boc)-OH, provided the deprotected amine, H-Lys(Boc)-OPMB (5), shown in Scheme 2. The urea precursors (4) and (5) were then coupled together as reported to form the protected form of DCL.

A key modification in our synthesis of DCL was the complete deprotection of all the protecting groups on the urea using TFA in dichloromethane to yield the fully deprotected DCL (7), shown in Scheme 2. Similar to the use of carbobenzyloxy (Cbz) for differential protection of the lysine by Maresca et al.\cite{21} this avoided the selective deprotection of Boc in the presence of Pmb groups. As applied to our modular method, this also avoided treatment by strong acid which would degrade the Gd$^{3+}$ and placeholder chelates after their incorporation into the TMIA.

Initially, a two-step procedure was utilized to attach the substrate linker, DSS, onto the metal-chelated imaging module, followed by conjugation of the DCL in its protected or deprotected form in the last step. However, for our approach, a preferred method was devised in which DCL, in its deprotected form (7), was coupled first to the linker DSS to form the targeting module (8) directly, as shown in Scheme 2.

A similar approach to the targeting module, DCL-DSS (8), was previously reported in the syntheses of similar PSMA targeted MRI agents, and dual agents for OMI/SPECT.\cite{10, 12, 21} In those reports, a two-step procedure couples the tri-PMB or tributyl protected DCL to the DSS. In the approach in Scheme 2, targeting module (8) is synthesized in one step from the entirely deprotected DCL (7). We also found that the activated DCL-DSS module could be conveniently purified by reverse-phase HPLC under mildly acidic conditions.

The modular synthesis of the TMIA for MRI (9a) was then completed in one convergent step by coupling imaging module (3) to targeting module (8) as shown in Scheme 3. This TMIA-containing DOTA-chelated Gd$^{3+}$, is analogous to the...
single Gd agent described earlier in the report on PSMA-targeted contrast agents for MRI.[10]

The nonradioactive, model TMIAs for PET were synthesized by first forming the analog containing the La$^{3+}$ placeholder metal (9b) by the same convergent step, followed by transmetalation by one of the two step methods described earlier for imaging module (2c). This involved a slow, demetalation in mild acid, followed by rapid chelation in solution for Cu$^{2+}$ (10b) and Y$^{3+}$ (10d) or on a SPE cartridge for Ga$^{3+}$ (10a) and In$^{3+}$ (10c) to produce the model TMIAs for PET/SPECT.

As in the transmetalation reactions described for the imaging module (2c), complete removal of the placeholder, and 100% re-metalation by the four metals was verified to the detection limit by LCMS.

Relaxivity ($r_1$) values of four compounds including two Fmoc-protected modular intermediates (2a and 2b), the final targeted molecular imaging agent (9a), and a sample of Gd-DOTA (Dotarem$^\text{TM}$) were measured at 40 MHz. The $r_1$ values are presented in Table 1 showing that the Gd-containing modules and the TMIA have $r_1$ values comparable to Gd-DOTA (Dotarem$^\text{TM}$). TMIA (9a) was also measured at 200 MHz to yield a similar $r_1$ of 4.26 mm$^{-1}$ s$^{-1}$ (see the Supporting Information).

In summary, a modular method for the synthesis of TMIAs was applied to the synthesis of PSMA-targeted imaging agents for MRI, PET, and SPECT of prostate cancer. In the approach, metals utilized for MRI (Gd$^{3+}$), or placeholders (La$^{3+}$, Ce$^{3+}$) for radioactive metals for PET (Cu$^{2+}$, Ga$^{3+}$, In$^{3+}$, and Y$^{3+}$) were chelated to DOTA and placed on the side chains of protected lysines early in the synthesis to form imaging modules. In one convergent step, these were coupled directly to a targeting module consisting of the PSMA-binding urea DCL bonded to an activated linker.

This modular method provides PSMA-targeted agents for MRI directly with no further transmetalation or deprotection steps. By using placeholder analogues of the PET agents, all synthetic steps leading to the targeted imaging agent can be accomplished without the constraints of time and safety aspects of handling radiochemicals. Moreover, the agent containing a placeholder can be transported and stored until a small portion of it is needed for transmetalation. The very mild acid conditions for transmetalation are amenable to preparation of PET agents in a clinical setting.

Of the clinically relevant applications for prostate cancer, the method should be applicable to the synthesis of related PSMA TMIAs such as the tailor-made $^{68}$Ga-labeled Glu-urea-Lys-(Ahx)-HBED-CC, which contains a lipophilic amino acid bonded to a linker between the DOTA-based PET imaging moiety and the DCL targeting moiety.[32] It could also be utilized to synthesize targeted radioligand therapeutic agents, such as the DOTA based $^{177}$Lu-PSMA-617. The method of preparation for these examples, currently in clinical use in patients with metastatic castration-resistant prostate cancer, and closely related ana-

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**Table 1.** Relaxation rates of contrast agents.

<table>
<thead>
<tr>
<th>Contrast agent</th>
<th>$r_1$ (mm$^{-1}$ s$^{-1}$) 1 T</th>
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<tbody>
<tr>
<td>F-Lys(Gd-DOTA)-NH$_2$ (2a)</td>
<td>4.88</td>
</tr>
<tr>
<td>F-dLys(Gd-DOTA)-OH (2b)</td>
<td>4.45</td>
</tr>
<tr>
<td>DCL-DSS-Lys(Gd-DOTA)-NH$_2$ (9a)</td>
<td>4.66</td>
</tr>
<tr>
<td>Gd-DOTA (Dotarem$^\text{TM}$)</td>
<td>4.22</td>
</tr>
</tbody>
</table>

logies now in preclinical studies, should be amenable to this convergent, modular method.\textsuperscript{24}

In the broader scope, the ability to conjugate a targeting agent in the last steps under mild conditions offers the use of a given modular imaging system for a wide variety of targeting systems. The imaging modules may be utilized in standard peptide coupling reactions and conjugated directly or by linkers to targeting agents in the final steps of synthesis. The mild conditions in the method are particularly amenable to targeting groups that are acid sensitive as it avoids the use of harsh acid in the final steps synthesis. We hope that others find the modular method useful in the synthesis of a wide variety of TMIs.

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**Conflict of interest**

The authors declare no conflict of interest.

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