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Solid Phase Synthesis of Short Peptide-Based Multimetal Tags for Biomolecule Labeling

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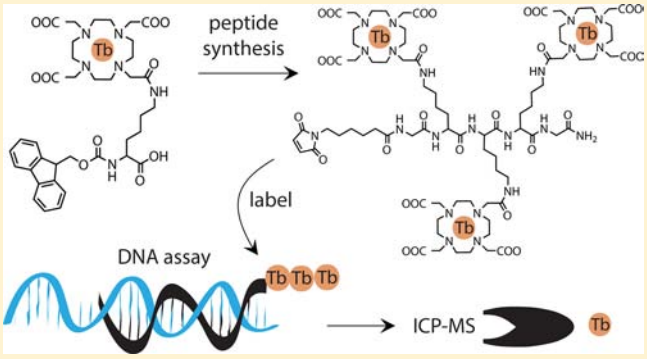
Supporting Information

ABSTRACT: We describe an unprecedented solid phase peptide synthesis (SPPS) of short peptide-based multimetal tags designated as elemental tags for the quantification of biomolecules via inductively coupled plasma mass spectrometry (ICP-MS). The macrocyclic chelator 1,4,7,10-tetraazacyclododecane *N,N′,N″,N‴*-tetra acetic acid (DOTA) was attached to the side chain of *N*-α-(9-fluorenylmethoxycarbonyl)-L-lysine (Fmoc-Lys-OH) and metalated with a lanthanide to provide a building block for Fmoc-based SPPS. Thereby, in contrast to existing strategies for the synthesis of DOTA–peptide conjugates, an already metalated DOTA-amino acid was used as a building block for SPPS. The DOTA-lanthanide complex was stable throughout the whole SPPS, even during the final cleavage in concentrated trifluoroacetic acid. This indicates that the strategy to first metalate the Fmoc-Lys(DOTA)-OH and to utilize the metal coordination to protect the carboxyl groups of DOTA offers an alternative to conventional synthetic routes using *tert*-butyl protected DOTA. Several small peptides containing up to four metal ions were synthesized, among them peptides carrying defined metal sequences consisting of two different lanthanides. The peptides were N-terminally maleimide-functionalized, thus introducing a moiety for conjugation to thiol-bearing biomolecules. The final objective of this work was the signal enhancement in ICP-MS-based DNA quantification assays. To evaluate the performance of the multimetal peptide tags in assay, they were applied to label thiol-modified 15mer DNA oligonucleotide probes. These served as reporter probes in a model sandwich-type hybridization assay. Thereby, we found that the ICP-MS signal increased linearly with the number of lanthanide ions attached to the reporter probe.



INTRODUCTION

The labeling of biomolecules with lanthanide-chelating tags, thus incorporating a characteristic heteroatom, enables the application of inductively coupled plasma mass spectrometry (ICP-MS) for detection and quantification. ICP-MS is a highly sensitive, reliable, and robust technique that is almost independent from analyte structure. The sample can be introduced as a solution either by direct infusion or in combination with separation techniques, e.g., liquid chromatography or capillary electrophoresis. Another possibility is sampling from surfaces of polyacrylamide gels, blotting membranes, microarrays, and tissue sections using laser ablation ICP-MS. In general, the labeling with lanthanide is achieved by bifunctional metal-chelating ligands offering both stable complexation of the metal and an additional functionality to covalently attach the tag to the target biomolecule.[1] Derivatives of the macrocyclic chelator 1,4,7,10-tetraazacyclododecane *N,N′,N″,N‴*-tetra acetic acid (DOTA) are frequently used for this purpose. Lanthanides are well suited for labeling: they are rare in biological samples, they are heavy elements not prone to isobaric interferences in ICP-MS, and they offer a wide choice of element and isotope masses for multiplexed analyses without signal overlapping. Lanthanide tags in combination with ICP-MS detection were applied to peptide and protein quantification by direct labeling,[2−4] and they were employed to label antibodies for ICP-MS based immunoassays,[5] including immunohistochemistry,[6,7] immunomicroarrays,[8,9] and mass cytometry.[10−12] Recently, efforts were made to extend the concept to sequence specific nucleic acid quantification by using lanthanide-labeled oligonucleotides as reporter probes in DNA assays.[13−15] In summary, the bioanalytes are labeled either directly, by formation of a covalent bond to the metal tag, or indirectly, by highly specific noncovalent interactions with the metal-labeled antibodies or oligonucleotide probes. The sensitivity of the ICP-MS-based quantification methods can be improved with tags containing more than one metal atom. This was previously shown with multiple DOTA-functionalized polymeric tags.[16] Lou et al. synthesized copolymers composed of *N,N*-dimethylacrylamide and *N*-acryloxysuccinimide with a narrow polydispersity carrying about 33 DOTA ligands per chain and applied them in immunoassay.[16] Further classes of metal-chelating polymers

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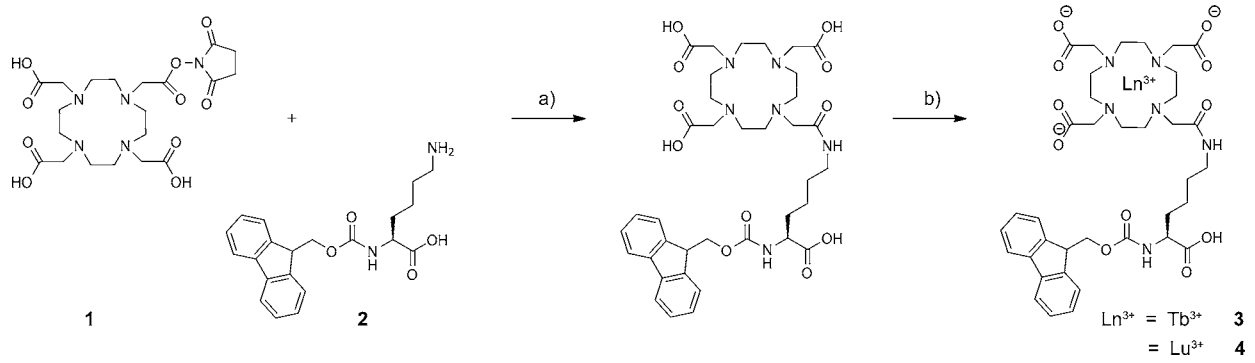
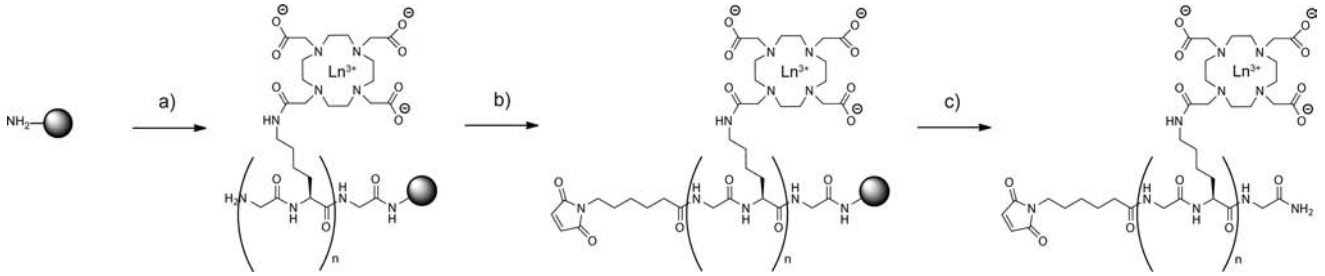
Scheme 1. Synthesis of the Metalated DOTA-Lysin Derivatives 3 and 4[a]



[a]Reagents and conditions: (a) 0.7 M TEAB/CH_3CN (1:1), pH 8.1, 3 h, 0 °C − r.t.; (b) $TbCl_3$ or $LuCl_3$, 0.5 M NH_4OAc/CH_3CN (4:1), pH 6.8, overnight, r.t.

Scheme 2. Solid-Phase Peptide Synthesis of the DOTA−Peptide Conjugates 5−10[a]



[a]Reagents and conditions: (a) Rink amide resin, Fmoc-amino acids (**3**, **4**, Fmoc-glycin), HCTU, HOBT, DIPEA; (b) maleimide hexanoic acid, HCTU, HOBT, DIPEA; (c) TFA/triisopropylsilane/H_2O (90:5:5), 1 h, r.t.

binding up to 68 metal ions on average were created by the same group and used for mass cytometry.[17−19] In other studies, those metal-chelating polymers attached to antibodies were applied in Western blot immunoassays in conjunction with laser ablation ICP-MS detection. A 20-fold rise in signal was observed with polymeric tags, compared to single lanthanide labels, when quantifying plant thylakoid proteins with this technique,[20] whereas another work mentioned limitations due to increasing background.[21] In the context of our work in the development of ICP-MS based DNA quantification approaches, we are interested in multiple DOTA-lanthanide-functionalized tags to improve the assay sensitivity. For this special application several properties of the tag are desirable: The tag, which is attached to a short oligonucleotide probe complement to the target DNA sequence, should not disturb hybridization of the probe to the target DNA. Furthermore, it should be hydrophilic to keep nonspecific binding low. Heat stability of the structure is preferred, since (repeated) heat denaturation steps or incubation at elevated temperature is often a part of DNA quantification methods. To ensure accurate quantification, monodispersity of the multimers is advantageous. The objective of the present study was to explore the capability of solid phase peptide synthesis (SPPS) to prepare peptide-based multimetal tags with DOTA ligands coupled to amino acid side chains. The degree of multimerization achievable with this method is limited and SPPS will result in oligomeric rather than polymeric tags, but the SPPS concept allows control of the number of lanthanide ions introduced and variation of the characteristics of the tag, e.g., its solubility, by choosing the amino acid sequence accordingly; in addition, even different lanthanide ions can be used for mixed-label tags. Strategies for the synthesis of DOTA−peptide conjugates, aimed as targeted radiopharmaceuticals and magnet resonance imaging (MRI) contrast agents, have been reviewed by De Leon-Rodriguez and Kovacs.[22] Reported SPPS pathways include the coupling of DOTA-tris(*tert*-butyl)-ester to the peptide N-terminus, directly onto the resin (C-terminal), or to the side chains of partially deprotected peptides. Besides the tris(*tert*-butyl)-ester, as the most common protected DOTA derivative, some research groups employed allyl-protected DOTA and DOTA-tris-(methyl)- and tris(benzyl)-ester.[22] Furthermore, DOTA-tris-(*tert*-butyl)-ester coupled to Fmoc-lysine, Fmoc-phenylalanine, and Fmoc-alanine derivatives, and used as SPPS building block were described.[23−25] There are also a few publications on the synthesis of peptides containing multiple DOTA-metal ligands. Boros et al. incorporated up to three DOTA-Gd into a polypeptide chain.[25] Overoye-Chan et al. attached four DOTA-Gd to the termini of a fibrin-binding peptide-based contrast agent by combination of solid phase and solution techniques.[26] The reported methods have in common that metalation is the last synthesis step. In the present work, we show an alternative way employing a metalated SPPS building block.

RESULTS AND DISCUSSION

Synthesis of Fmoc-Lys(DOTALn)-OH. In a first step, a metalated amino acid derivative, designated as a building block for SPPS, was synthesized (Scheme 1). The free side chain amine group of Fmoc-Lys-OH **2** was conjugated with DOTA via amide-bond formation using the *N*-hydroxysuccinimide ester, DOTA-NHS **1**. Reaction conditions were adapted and optimized from ref 3. Reaction was performed in TEAB buffer/CH_3CN at pH 8.1. Under these conditions, a 2-fold excess of

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Table 1. Characterization and Yields of the DOTA−Peptide Conjugates (5−10)

peptide	peptide sequence	calculated m/z[a]	found m/z[a]	synthetic yield %[b]	overall yield %[c]
5	MalGK(Tb)GK(Tb)G	861.767 $[M+2H]^{2+}$	861.767	83	51
6	MalGK(Tb)GK(Tb)GK(Tb)G	817.247 $[M+3H]^{3+}$	817.249	64	30
7	MalGK(Tb)GK(Tb)GK(Tb)GK(Tb)G	794.987 $[M+4H]^{4+}$	794.987	66	18
8	MalGK(Tb)K(Tb)K(Tb)G	779.233 $[M+3H]^{3+}$	779.230	(73)[d]	*d*
9	MalGK(Lu)GK(Tb)G	869.775 $[M+2H]^{2+}$	869.777	80	40
10	MalGK(Tb)K(Lu)K(Tb)G	784.571 $[M+3H]^{3+}$	784.574	(58)[d]	*d*

[a]Monoisotopic mass. [b]Obtained by Fmoc-monitoring after coupling the last Fmoc-Glycin and related to resin-NH_2 amount. [c]Obtained by weighing after RP-HPLC purification and related to resin-NH_2 amount. [d]Impurified with deletion sequence.

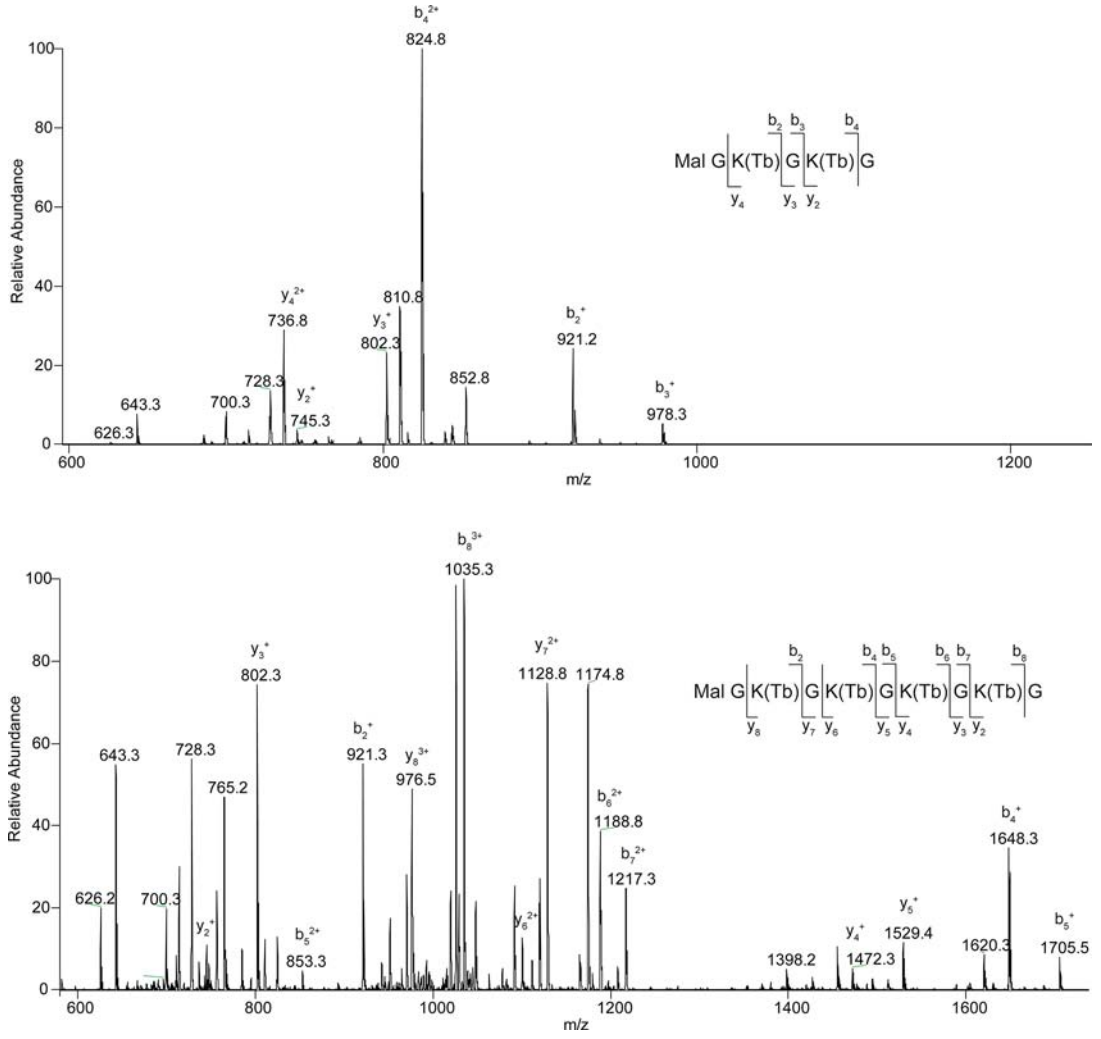

Figure 1. CID spectra of the doubly charged molecular ion of peptide **5** (top) and of the triply charged molecular ion of peptide **7** (bottom); m/z values of fragment ions corresponding to the b-series and y-series are indicated.

the expensive reagent DOTA-NHS was sufficient to ensure complete conversion of the Fmoc-Lys-OH. Subsequently, Fmoc-Lys(DOTA)-OH was metalated with Tb or with Lu in NH_4OAc buffer/CH_3CN at pH 6.8 to give the corresponding charge-neutral complexes **3** and **4**. After HPLC purification the metalated products **3** and **4** were obtained in 73% and 67% overall yield, respectively. The purity of the products was verified by HPLC-MS analysis. The amount of nonmetalated Fmoc-Lys(DOTA)-OH was either not detectable or less than 0.2%, when estimated from peak areas of the corresponding molecular ions in extracted ion chromatograms.

Whereas unmasked DOTA is only slightly soluble in organic solvents used for peptide synthesis, it becomes compatible with SPPS by conjugation to Fmoc-Lys-OH and metalation.

Synthesis of the Multimetal-Peptides. Peptide synthesis was performed on a Rink amide resin following standard Fmoc-protocol using HCTU, HOBT, and DIPEA for activation (Scheme 2). Short peptides were synthesized containing 2, 3, and 4 Lys(DOTATb) and Gly as a spacer (Table 1 **5**, **6**, **7**). Maleimide hexanoic acid was added as the last building block to the N-terminus of the peptides, thus providing a reactive moiety to attach the multimetal peptide to thiol-bearing biomolecules. The proper HPLC purification and drying of

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the Fmoc-Lys(DOTATb)-OH was a prerequisite for its reactivity. Yields of each coupling step were obtained by measuring the UV-absorption of the fulvene-piperidine adduct after Fmoc removal. Coupling yields for Fmoc-Lys(DOTATb)-OH coupled to Gly were 80−99%, when applying 4-fold excess of Fmoc-Lys(DOTATb)-OH. Coupling reactions of Fmoc-glycin to Lys(DOTATb) were quantitative.

When trying to synthesize a sequence with three Lys(DOTATb) coupled directly to each other (**8**), we observed in the case of incomplete coupling that for deletion sequences ending with Lys(DOTATb) the acetylation of the unreacted amino groups was not sufficiently effective, even when the acetylation step was prolonged to 15 min, repeated twice, or performed with an alternative capping solution Ac_2O/DIPEA/DMF (5:5:90 v/v/v). This incomplete capping resulted in a mixture of the target peptide and deletion sequences both maleimide functionalized and, therefore, difficult to separate. As on the other hand acetylation after Gly was complete, it seemed advantageous to position a Gly spacer before each Lys(DOTATb). Upon completion of the synthesis, the peptides were cleaved from the resin with concentrated TFA, precipitated in ether, and purified by HPLC. To verify the identity of the product peptides, the exact mass (ESI-HRMS) and the fragmentation pattern (ESI-MS/MS (CID)) of the molecule ion were examined. Characteristic y- and b-series type fragment ions confirmed the expected structure (Figure 1 and Supporting Information Figure S24). Some small fragment ions were typical for peptides containing Lys(DOTATb) residues and Gly-Lys(DOTATb), i.e., m/z 626, m/z 643 and m/z 700, m/z 728, respectively.

When using metalated DOTA as part of a building block, instead of DOTA with protected carboxyl groups, deprotection is not needed. Furthermore, metalated DOTA is less sterically demanding and hydrophobic than *tert*-butyl-protected DOTA. De Leon-Rodriguez et al. reported problems with coupling the next amino acids beyond tris(*tert*-butyl)-protected DOTA-amino acids.[24] The more reactive *N*-α-Fmoc-pentafluorophenyl ester amino acid derivatives were required for the following amino acids. Boros et al. had to apply forcing conditions to achieve product formation when coupling a tris(*tert*-butyl)-protected DOTA-alanine derivative into a peptide sequence between two Gly.[25]

Stability of the Chelate Complex During SPPS. The HPLC-MS analysis of the crude product showed besides the main product peptide the expected acetylated short length side products, but no evident side reactions of the DOTA carboxyl groups. Most surprisingly, the DOTA-metal complex resists the final cleavage step in 90% TFA. Loss of metal was observed to a small extent of about 1−2% per incorporated Lys(DOTATb), when estimated in a semiquantitative manner from peak areas of the corresponding peptide molecular ions in extracted ion chromatogram (Figure 2) and defined as $\text{area}_{(\text{product-Tb+3H})}/(\text{area}_{(\text{product})} + \text{area}_{(\text{product-Tb+3H})}) \times 100\%$. This side products with loss of one Tb could be separated from the desired product by HPLC purification. The loss of metal might become crucial when synthesizing longer peptides; as the number of Lys(DOTATb) per peptide increases there is a greater probability to lose one metal ion. To address these limitations, we are currently exploring milder cleavage conditions, employing another resin, and the first results indicated that the loss of metal might be circumvented (data not shown). However, the complex stability for the DOTA-peptides in 90% TFA was unexpectedly high. We observed that although thermodynamically favored the acid promoted demetalation of the DOTA-peptides took place over several weeks when the peptides (after separation from the resin) were further incubated in the 90% TFA cleavage solution at room temperature and that the demetalation proceeded more slowly when the TFA concentration was lowered to 45% by addition of water (data not shown). De Silva et al. applied DOTA-Eu-labeled peptides in a ligand binding assay based on dissociation-enhanced lanthanide fluorescence.[27] The complete release of europium from the DOTA chelate, as required before detection, was achieved by treating the complex for 2 h with 2 M HCl at 37 °C; experiments with TFA were not successful. We suppose that the different acid strengths might be one reason for the different effect of HCl and TFA on DOTA complex stability. Rates of dissociation of DOTA-lanthanide complexes in strong inorganic acids were determined by different authors.[28−30] Furthermore, it is well-known that the DOTA-lanthanide complex dissociation kinetics depends on the size of the lanthanide ion, i.e., the complex stability tends to increase with the decrease in the ionic size within the series of lanthanides.[28,31−33]

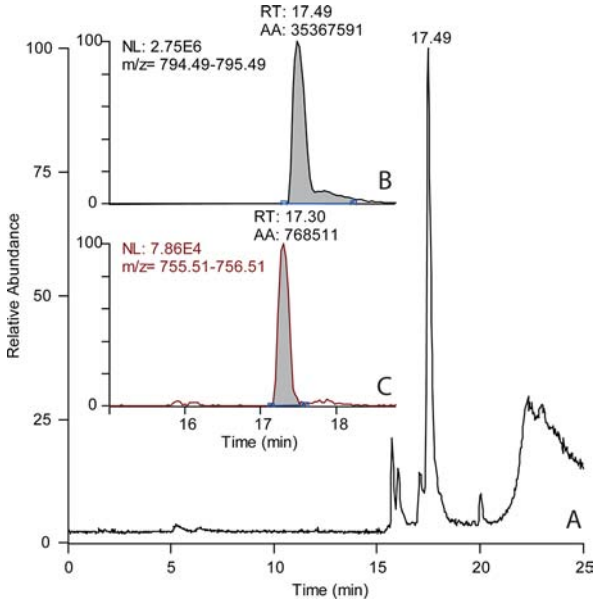

Figure 2. HPLC-HRMS chromatogram of the crude peptide **7** obtained after cleavage and precipitation (HPLC system (A2), positive ESI, m/z 400−4000). Total ion chromatogram (A) and extracted ion chromatograms of the doubly charged molecular ions of peptide **7** (B) and peptide **7** with loss of one Tb (C). Abbreviations: RT = retention time in min; AA = peak area; NL = normalized largest (signal intensity); m/z = m/z range of extracted ion chromatogram.

Different Lanthanide Ions within One Multimetal Peptide Sequence. The presented SPPS method allows the site-specific introduction of different lanthanides into one peptide sequence. We synthesized two peptides containing both Tb and Lu (**9**, **10**). HPLC-MS analysis of the crude SPPS products revealed that they consisted of the desired metal sequences only (Supporting Information). This is proof for the synthetic pathway starting with metalated amino acids, because metalation with a Tb/Lu solution after completion of the peptide synthesis would result in a mixture of peptides differing in their metal sequence. In the future, multimetal coded peptide tags might be useful to expand multiplexing capabilities, e.g., for application as mass tags in molecular mass spectrometry based

proteomic approaches. For application in ICP-MS based assays, multimetal coding might at least in some cases also be achieved by simply mixing labeling reagents that contain different metals as demonstrated by Bodenmiller et al., who used 7 differentially metalated tags to compose a barcode of 128 combinations applied to label whole cells in 96-well plates prior to analysis by mass cytometry.[11] In principal, multimetal coding should be realizable in a straightforward way, when the differentially labeled analytes are spatially separated from each other or are analyzed in a time-resolved manner. In contrast, simultaneous detection of multiple analytes differentially labeled with coded tags is crucial, because degeneracy of the code might occur, i.e., different analyte combinations might give the same test result. An interesting combinatorial coding scheme, developed for qualitative tests, circumventing this problem and ensuring unambiguous results for large-scale multiplexing was introduced by Rajagopal et al. and applied to multiplexed Taqman PCR assays, using a few fluorophores only, but labeling each sequence with a certain mixture of these fluorophores.[34]

Application of the Multimetal-Peptide Tag for Signal Enhancement in ICP-MS-Based DNA Assay. The utility of the lanthanide-bearing peptides as multimetal tags for signal enhancement in ICP-MS based DNA quantification was examined in a basic sandwich-type hybridization assay, which was described in depth elsewhere.[15] Thiol-modified 15mer DNA probes were labeled with either 1, 2, or 4 Tb. Fivefold excess of the maleimide functionalized DOTA−peptide conjugates was sufficient to achieve complete labeling of the oligonucleotides.

In assay, a single-stranded 49mer target DNA formed hybrids with two complement DNA probes, the metal-labeled reporter probe and a biotinylated capture probe. These hybrids were isolated from an excess of reporter probes in streptavidin coated microtitration wells, eluted with nitric acid, and the Tb amount was analyzed by ICP-MS. Assay calibration curves comprising 7 levels of target DNA amount in the range 10−1000 fmol are shown in Figure 3. The slope of the regression lines increased linearly with the number of Tb atoms attached to the reporter probes; thus, the peptide tags seemed not to hamper hybridization.

The limit of detection (LOD) is a function of response and background. The blank value of the assays is caused by the Tb background of ingredients, lab environment, and ICP-MS instrument, and by nonspecifically bound reporter probes, where the signal from the latter as well is amplified by the multimetal tags. For application in such an assay a multimetal tag is only then beneficial, when the part of the background signal arising from nonspecifically bound probes is low and disposition of the multimetal tag to nonspecific binding is not much higher than that of the single label. A moderately hydrophilic nature of the DOTA−peptide tags was indicated by reversed phase HPLC analysis. Retention time decreased with increasing number of DOTA ligands. Results of an experiment studying the impact of the multiple labeling on assay background are summarized in Table 2. The assay blank value increased with the number of Tb atoms per probe, but the comparison with assay blank values performed without probes revealed that the tendency to bind nonspecifically decreases for the multiple labeled probes. The theoretical LOD was calculated to 2.3 fmol DNA target, when applying single labeled probes, and 0.4 fmol DNA target with the 4-fold labeled probes. Thus, the assay sensitivity was improved by the use of the multimetal peptide tags.

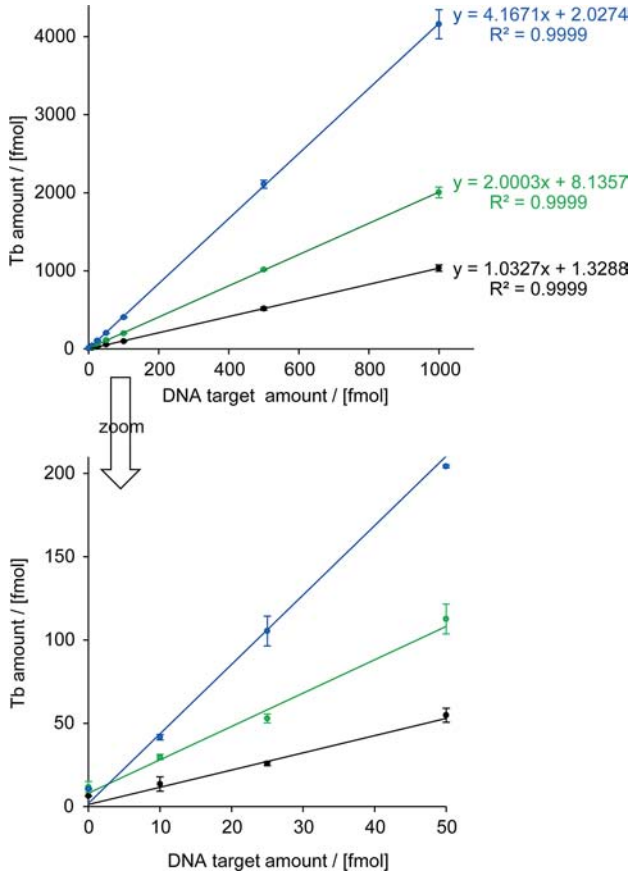

Figure 3. Quantification of DNA in a sandwich-type hybridization assay in conjunction with ICP-MS detection. Determined amount of Tb as a function of the target DNA amount. Comparison of three assay calibration curves: black, performed with reporter probes labeled with one DOTATb; green, reporter probes labeled with the DOTA−peptide tag **5** carrying two DOTATb ligands; blue, reporter probes labeled with the DOTA−peptide tag **7** carrying four DOTATb ligands. The error bars represent the standard deviation of independent replicates (n = 3).

Table 2. Quantification of DNA in a Sandwich Hybridization Assay Using Multiple Lanthanide-Labeled Probes and ICP-MS Detection[a]

	assay blank value (n = 6)		slope of calibration curve	LOD DNA = 3.3SD/m
assay blank value	median/ [fmol Tb]	±SD/ [fmol Tb]	m/[fmol Tb/ fmol DNA]	LOD/[fmol DNA]
without probes	3.1	0.5		
probes with one Tb	4.5	0.7	1.03	2.3
probes with two Tb	5.3	0.8	2.00	1.4
probes with four Tb	6.9	0.5	4.17	0.4

[a]Influence of the multiple-labeled probes on assay blank values, response, and LOD.

These LOD values were comparable in range with LOD values obtained with various fluorescence tags in a similar simple sandwich-type DNA assay system.[35] The elemental labeling strategy can be combined with molecular biological amplification techniques to further improve the sensitivity.[14,15]

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We already demonstrated the suitability of the lanthanide-labeled reporter probes in a ligase chain reaction based assay.[15] Similar experiments applying the multimetal tags instead of the single labeled tag were also successful, indicating the compatibility of the multimetal tags with temperature cycling and ligase reaction (data not shown). The special potential of the elemental labeling for DNA quantification when compared to the widespread fluorescence labeling lies in the inherent high multiplexing capability,[13] disadvantageous are the instrument costs and the limitation to heterogeneous DNA assay formats.

CONCLUSIONS AND FUTURE PROSPECTS

In summary, we reported the preparation of multiple lanthanide-labeled tags by SPPS using a metalated amino acid building block. As a proof-of-concept, we synthesized peptides bearing up to four lanthanide ions. The tags were then conjugated to oligonucleotide reporter probes and applied to sequence specific DNA quantification via ICP-MS. The multilanthanide label compared to single-lanthanide label resulted in the expected increase of signal and in a decrease of the LOD of our model sandwich-type hybridization assay. The most interesting finding is the stability of the DOTA metal complex under (harsh) organic synthesis conditions. At least for incorporation of stable metal isotopes, the strategy to first metalate the DOTA and to employ the metal coordination to protect the carboxyl groups might offer a straightforward alternative to the established method which involves the use of *tert*-butyl protected DOTA derivatives and the metalation as final synthesis step. This is a promising result not only for application in ICP-MS-based biomolecule analysis. Taking advantage of the paramagnetic and luminescent properties of some lanthanides, lanthanide-chelating DOTA–peptides found application as targeting vector for magnetic resonance imaging,[e.g.,25,26,36,37] and as luminescence probes to study protein functions.[e.g.,27,38,39] Furthermore, the described synthetic pathway enabled access to the first synthesis of peptides containing a certain sequence of different lanthanides.

EXPERIMENTAL PROCEDURES

Materials. 1,4,7,10-Tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid mono(*N*-hydroxysuccinimidyl) ester (DOTA-NHS, **1**) was purchased from Chematech (Chematech, Dijon, France); *N*-α-(9-Fluorenylmethoxy-carbonyl)-L-lysine (Fmoc-Lys-OH, **2**) was received from Novabiochem (Merck, Darmstadt, Germany). Tentagel R Ram resin (0.18 μmol mg^{-1}) was acquired from Rapp Polymere (Rapp Polymere, Tübingen, Germany). DMF and DCM were peptide synthesis grade (Carl Roth, Karlsruhe, Germany). Oligonucleotides were obtained in HPLC quality (Eurofins MWG biotech, Ebersberg, Germany). The 49mer **D1** (5′-GGTCGGTGCATTAATAAG-TGGAATATAATTTTTCAAATCCATATCTACA-3′) served as single-stranded target DNA. The sequences and modifications of the oligonucleotide probes were **D2** (5′-thiolhexyl/GTAGATATGGATTTG-3′) and **D3** (5′-ATTAATGCACC-GAC/biotin-3′). Streptavidin coated microwell strips Strepta-Well (regular binding capacity) were from Roche Diagnostics (Roche, Mannheim, Germany). Nitric acid was purchased in optima grade (Fisher Scientific, Loughborough, UK). Multielement lanthanide standard solution was obtained from Alfa Aesar (Alfa Aesar, Karlsruhe, Germany) and single-element Bi standard solution from Carl Roth (Carl Roth, Karlsruhe, Germany). Water used for all experiments was purified with an USF Elga Purelab Plus system (ELGA, Marlowe, UK).

Chromatography. Purification by HPLC-UV (P1, P2, P3) was performed with a SpectraSystem (Thermo separation products, San Jose, CA, USA). System P1: column Vydac RPC18 201TP1022, 250 × 22 mm, 10 μm, UV 262 nm, flow 5 mL min^{-1}, solvent (a) 50% CH_3OH, 49.9% H_2O, 0.1% FA; (b) 90% CH_3CN, 9.9% H_2O, 0.1% FA; gradient (a): 0 min 95%, 10 min 95%, 30 min 10%, 40 min 10%, 45 min 95%, 60 min 95%. System P2: column Varian Polaris 5 C18 Ether, 250 × 4.6 mm, 5 μm, UV 210 nm, flow 0.8 mL min^{-1}; solvent (a) 2.5% CH_3OH, 97.4% H_2O, 0.1% FA; (b) 99.9% CH_3CN, 0.1% FA; gradient (a): 0 min 100%, 10 min 100%, 30 min 50%, 40 min 50%, 50 min 100%, 60 min 100%. System P3: column Phenomenex Gemini-NX, 50 × 4.6 mm, 3 μm, UV 260 nm, flow 0.8 mL min^{-1}, solvent (a) 95% 25 mM TEAA in H_2O (pH 5.5), 5% CH_3CN; (b) 100% CH_3OH; gradient (a): 0 min 95%, 20 min 80%, 29 min 10%, 31 min 10%, 33 min 95%, 35 min 95%. Analytical HPLC-MS (A1, A2, A3) was performed with an Agilent 1200 series instrument (Agilent Technologies, Waldbronn, Germany) coupled to a Finnigan LTQ FT ULTRA mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). System A1: column Phenomenex Luna C18(2), 150 × 1 mm, 5 μm, flow 50 μL min^{-1}; solvent (a) 10% CH_3OH, 89.9% H_2O, 0.1% FA; (b) 90% CH_3CN, 9.9% H_2O, 0.1% FA; gradient (a): 0 min 99%, 10 min 99%, 40 min 10%, 50 min 10%, 55 min 99%, 70 min 99%. System A2: column Phenomenex Luna C18(2), 150 × 1 mm, 5 μm, flow 50 μL min^{-1}; solvent (a) 2.5% CH_3OH, 97.4% H_2O, 0.1% FA; (b) 90% CH_3CN 0.1% FA; gradient (a): 0 min 100%, 10 min 100%, 15 min 30%, 30 min 30%, 35 min 10%, 45 min 10%, 50 min 100%, 70 min 100%. System A3: column Supelco Discovery BIO Wide Pore C18, 100 × 1 mm, 3 μm, flow 40 μL min^{-1}; solvent (a) 99% 20 mM TEAA in H_2O (pH 5.5), 1% CH_3OH; (b) 10% 20 mM TEAA in H_2O (pH 5.5), 90% CH_3OH; gradient (a): 0 min 90%, 5 min 90%, 15 min 70%, 20 min 1%, 30 min 1%, 35 min 90%, 45 min 90%.

Mass Spectrometry. HPLC-HRMS measurements were performed on a Finnigan LTQ FT ULTRA mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). DOTA–peptide conjugates were analyzed in positive ionization mode; the electrospray voltage was 5 kV and the capillary temperature 275 °C. Scans in a mass range of m/z 400–4000 were performed in the Fourier transform ion cyclotron resonance cell of the mass spectrometer. For further characterization of the DOTA–peptide conjugates fragmentation experiments by collision induced dissociation (CID) were performed within the same run in the linear ion trap of the mass spectrometer. The most intense ions were selected in a data dependent manner, isolated with an isolation width of m/z 3.5, and fragmented applying a normalized collision energy of 35%, an activation time of 30 ms, and an activation Q of 0.25.

The ICP sector field mass spectrometer Element XR (Thermo Fisher Scientific, Bremen, Germany) was equipped with a MicroMist concentric nebulizer (Glass Expansion, West Melbourne, Vic., Australia) and a Twinnabar cyclonic spray chamber (Glass Expansion, West Melbourne, Vic., Australia). Sample uptake rate by self-aspiration was about 150 μL min^{-1}. Nebulizer gas flow was 1.7 L min^{-1}, plasma power was 1300 W. ^{159}Tb and ^{209}Bi isotopes were recorded in low resolution mode. Standard solutions for external calibration (1–1000 ng kg^{-1} per element in 3.5% (v/v) nitric acid) were prepared from multielement lanthanide standard solution. The samples and

the lanthanide standard solutions for external calibration were spiked with 100 ng kg^{-1} Bi internal standard.

Synthesis. *Preparation of Fmoc-Lys(DOTALn)-OH.* 1,4,7,10-Tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid mono(*N*-hydroxysuccinimidyl) ester (DOTA-NHS) **1** (19.08 mg, 25.1 μmol) was dissolved in 200 μL ice-cold H_2O/CH_3CN (1:1 v/v) and immediately added to a solution of *N*-α-(9-fluorenylmethoxycarbonyl)-L-lysine (Fmoc-Lys-OH) **2** (4.65 mg, 12.6 μmol) in 550 μL (1 M TEAB in H_2O)/CH_3CN (1:1 v/v); pH 8.1 was adjusted with TEA (0.1 M in CH_3CN). The reaction mixture was incubated for 3 h at room temperature with shaking. Thereafter, solvent and volatile buffer were removed by vacuum centrifugation and lyophilization. The dry residue was redissolved in 600 μL CH_3CN and 2400 μL 0.5 M NH_4OAc in H_2O. To metalate the complex, a solution of the lanthanide chloride, e.g., $TbCl_3$ (13.26 mg, 50.0 μmol), in 27 μL 0.04 M HCl/0.5 M NH_4OAc in H_2O (1:1 v/v) was added and the reaction mixture was incubated at room temperature with shaking overnight or for at least 3 h. Subsequently, the mixture was lyophilized, dissolved in 600 μL CH_3OH/CH_3CN/H_2O/FA (50:10:39.9:0.1 v/v/v/v), and, after removal of insoluble solids by centrifugation, purified by reversed phase HPLC (P1). The product fraction, eluting as a double peak, was lyophilized to obtain Fmoc-Lys(DOTATb)-OH **3** (8.38 mg, 9.21 μmol, 73%) as a white powder. Likewise, Fmoc-Lys(DOTALu)-OH **4**, an analogue metalated with lutetium, was prepared with 67% yield. The crude and the purified products were characterized by HPLC-HRMS (HPLC (A1)): **3** calcd *m*/*z* 911.263 $[M + H]^+$; found *m*/*z* 911.261; **4** calcd *m*/*z* 927.279 $[M + H]^+$; found *m*/*z* 927.276. ^1H and ^{13}C NMR spectra were taken using an Avance II 500 MHz spectrometer (Bruker, Billerica, MA, USA). NMR characterization of **3** yielded broad unresolved signals, because of the highly paramagnetic properties of Tb. Better results were obtained for the diamagnetic Lu analogue **4**: ^1H NMR (500 MHz, d_6-DMSO): δ = 9.55, 8.17 (s, 1H), 7.89 (d, J = 7.54 Hz, 2H), 7.75 (dd, ^4J = 2.76, ^3J = 7.39 Hz, 2H), 7.67 (m, 1H), 7.42 (t, J = 7.48 Hz, 2H), 7.33 (t, J = 7.45 Hz, 2H), 4.23 (m, 3H), 3.92 (m, 1H), 3.61 (m, 2H), 3.33 (m, 9H*), 3.17 (m, 2H), 2.81 (m, 5H), 2.64 (m, 4H), 2.36 (m, 4H), 1.67 (dt, ^2J = 34.92, ^3J = 7.84 Hz, 2H), 1.48 (m, 2H), 1.35 (m, 2H) ppm; ^{13}C NMR (125 MHz, d_6-DMSO) δ = 175.80, 174.68, 164.04, 156.83, 144.50, 144.46, 141.32, 128.20, 127.64, 125.90, 120.60, 65.81, 60.99, 54.01, 48.21,46.68, 30.46, 27.88, 22.78 ppm. (The * denotes overlapping with signal from residual water.)

Solid Phase Peptide Synthesis. SPPS was performed manually on a 1 μmol scale using 2 mL polyethylene syringe reactors equipped with a fritted disc. After initial swelling in DMF for 30 min, Tentagel R Ram resin (5.55 mg, 1 μmol H_2N-) was treated twice with 0.5 mL DMF/piperidine (4:1 v/v) for 2 min to remove Fmoc protecting group and was then washed with DMF (3× 1 mL), DCM (3× 1 mL), DMF (3× 1 mL). For coupling of Fmoc-Gly-OH, 8 equiv Fmoc-Gly-OH, 8 equiv HOBT, and 7.2 equiv HCTU were dissolved in DMF and after addition of 24 equiv DIPEA transferred to the resin. The final concentration of Fmoc-Gly-OH in the coupling solution was 0.1 M. Equivalents were related to the actual amount of free amino groups (1 equiv) as determined from the previous Fmoc removal step (see below). For coupling of Fmoc-Lys-(DOTALn)-OH (**3**, **4**), 4 equiv of **3** (or of **4**), 4 equiv HOBT, and 3.6 equiv HCTU were dissolved in DMF/*N*-methylpyrrolidone (1:1 v/v); 12 equiv DIPEA was added. The final concentration of **3** (or of **4**) in the coupling solution was 0.1 M. Couplings were conducted for 60 min at room temperature. Following each coupling step the resin was washed as described above and residual free amino groups were acetylated with a solution of 1 mL Ac_2O/2,6-lutidine/DMF (5:6:89 v/v/v) for 2 min. Removal of the Fmoc protecting group was performed by treating the resin twice with each 0.5 mL DMF/piperidine (4:1 v/v) for 2 min. Both deprotection solutions were collected and combined to determine the amount of Fmoc, and thereby the coupling efficiency by measuring the absorbance of the fulvene−piperidine adduct at 301 nm (ε at 301 nm 7800 M^{-1} cm^{-1}) in a Specord 205 (Analytik Jena, Jena, Germany). As last building block maleimide hexanoic acid was coupled to the N-terminus, by treating with 8 equiv maleimide hexanoic acid, 8 equiv HOBT, 7.2 equiv HCTU, and 24 equiv DIPEA in DMF at room temperature for 45 min. This last coupling step was performed twice. The resin was then washed with DMF (6× 1 mL), DCM (6× 1 mL), and dried under vacuum. Final cleavage from the resin was carried out with 0.55 mL TFA/H_2O/triisopropylsilane (90:5:5 v/v/v) at room temperature for 1 h. The cleavage solution was precipitated in 10 volumes of cold diethyl ether and separated by centrifugation. The crude peptide was dissolved in H_2O/FA (99.9:0.1 v/v), freeze−dried, redissolved, and subjected to reversed phase purification (HPLC (P2)). After lyophilization the purified product was obtained as a white solid. Prior to use for multimetal labeling, the DOTA−peptide conjugates were dissolved in H_2O/FA (99.9:0.1 v/v), i.e., in acidified solution to prevent hydrolysis of the maleimide.

For long-term storage, the DOTA−peptides should be preferably stored in lyophilized form or as a stock solution in DMSO. The crude and the purified product peptides (**5**−**10**, Table 1) were characterized by HPLC-HRMS (HPLC (A2)).

Labeling of Thiol-Modified Oligonucleotides with DOTA−Peptide Conjugates. The thiol-modified 15mer DNA oligonucleotide **D2** was incubated with 3-fold molar excess of tris(2-carboxyethyl) phosphine (TCEP) for 30 min at 50 °C in 0.1 M phosphate buffer (pH 7.4) to reduce possible disulfide bonds. Subsequently, the maleimide functionalized DOTA−peptide conjugate **5** (or **7**) was added in 5-fold molar excess. The final concentration of the oligonucleotide in the reaction mixture was 100 μM. The reaction was allowed to proceed overnight at 37 °C. The product was purified by HPLC (HPLC (P3)), freeze−dried, redissolved in water, and stored at −20 °C until use. For comparison with the multiple labeled probes in assay DNA oligonucleotide **D2** was labeled with the established maleimide functionalized reagent **DOTA-Mal-Tb**[2] to obtain the single Tb-labeled reporter probe **D2-DOTA-Mal-Tb**, following the same labeling protocol.[15] The purified product conjugates were characterized by HPLC-HRMS (HPLC (A3)): **D2-DOTA-Mal-Tb** calcd *m*/*z* 1416.998 $[M-4H]^{4-}$; found *m*/*z* 1417.000; **D2**−**5** calcd *m*/*z* 1643.333 $[M-4H]^{4-}$; found *m*/*z* 1643.340; **D2**−**7** calcd *m*/*z* 2006.933 $[M-4H]^{4-}$; found *m*/*z* 2006.911. The final concentration of the Tb-labeled probes was determined by ICP-MS measurement of the Tb content as described above, diluted in 3.5% HNO_3 and spiked with Bi internal standard.

DNA Assay. The sandwich-type hybridization assay was performed as described elsewhere.[15] Different amounts of single-stranded target DNA **D1** (10−1000 fmol), 3 pmol metal labeled reporter probe (**D2-DOTA-Mal-Tb**, **D2**−**5**, or **D2**−**7**), and 2 pmol biotinylated capture probe **D3**, all in a total volume of 60 μL buffer A (0.1 M phosphate buffer (pH 7.2), 1 M NaCl, 0.01% (w/w) Tween 20), were heated to 93 °C for 3 min, and

cooled down to room temperature within 60 min to achieve hybridization. Subsequently, this mixture was given to the streptavidin-coated wells and filled up to 300 μL with buffer B (0.1 M phosphate buffer (pH 7.4), 1 M NaCl, 0.1% (w/w) BSA, 0.01% (w/w) Tween 20). The biotinylated hybrids were allowed to bind for 60 min with shaking at room temperature. Thereafter, the wells were rinsed twice with buffer B and once with buffer A. To liberate the immobilized hybrids, the wells were filled with 300 μL 13% (v/v) nitric acid each and heated to 97 °C for 30 min. The hot elute solution was transferred to a tube, diluted with water and spiked with Bi internal standard solution to give about 1.2 mL sample containing 3.5% (v/v) nitric acid and 100 ng kg^{-1} Bi. To correct for losses due to evaporation during heating, the weight of the added internal standard solution and of the total sample solution was used for calculations. Finally, the Tb/Bi response of the samples was obtained by ICP-MS measurement as described above.

■ ASSOCIATED CONTENT

Supporting Information

HPLC-UV and HPLC-HRMS data of compound **3**; copies of ^{1}H, ^{13}C NMR spectra of compound **4**; HPLC-UV traces and HPLC-HRMS data for the peptides (**5**−**10**); CID fragmentation pattern for the peptides **6**, **8**, **9**, **10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Ac_2O, acetic anhydride; CID, collision induced dissociation; DIPEA, *N*,*N*-diisopropylethylamine; DOTA, 1,4,7,10-tetraazacyclododecane *N*,*N*′,*N*″,*N*‴-tetra acetic acid; FA, formic acid; Fmoc, 9-fluorenylmethyloxycarbonyl; HCTU, *O*-(6-chlorobenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluorophosphate; HOBT, *N*-hydroxybenzotriazole; LOD, limit of detection; NHS, *N*-hydroxysuccinimide; SD, standard deviation; SPPS, solid phase peptide synthesis; TEA, triethylamine; TEAA, triethylammonium acetate; TEAB, triethylammonium bicarbonate; TFA, trifluoroacetic acid

■ REFERENCES

(1) Lattuada, L., Barge, A., Cravotto, G., Giovenzana, G. B., and Tei, L. (2011) The synthesis and application of polyamino polycarboxylic bifunctional chelating agents. *Chem. Soc. Rev. 40*, 3019−3049.

(2) Ahrends, R., Pieper, S., Kuehn, A., Weisshoff, H., Hamester, M., Lindemann, T., Scheler, C., Lehmann, K., Taubner, K., and Linscheid, M. W. (2007) A metal-coded affinity tag approach to quantitative proteomics. *Mol. Cell. Proteomics 6*, 1907−1916.

(3) Bergmann, U., Ahrends, R., Neumann, B., Scheler, C., and Linscheid, M. W. (2012) Application of metal-coded affinity tags (MeCAT): absolute protein quantification with top-down and bottom-up workflows by metal-coded tagging. *Anal. Chem. 84*, 5268−5275.

(4) Schwarz, G., Mueller, L., Beck, S., and Linscheid, M. W. (2014) DOTA based metal labels for protein quantification: a review. *J. Anal. At. Spectrom. 29*, 221−233.

(5) Careri, M., Elviri, L., and Mangia, A. (2009) Element-tagged immunoassay with inductively coupled plasma mass spectrometry for multianalyte detection. *Anal. Bioanal. Chem. 393*, 57−61.

(6) Hutchinson, R. W., Cox, A. G., McLeod, C. W., Marshall, P. S., Harper, A., Dawson, E. L., and Howlett, D. R. (2005) Imaging and spatial distribution of β-amyloid peptide and metal ions in Alzheimer's plaques by laser ablation−inductively coupled plasma−mass spectrometry. *Anal. Biochem. 346*, 225−233.

(7) Giesen, C., Mairinger, T., Khoury, L., Waentig, L., Jakubowski, N., and Panne, U. (2011) Multiplexed immunohistochemical detection of tumor markers in breast cancer tissue using laser ablation inductively coupled plasma mass spectrometry. *Anal. Chem. 83*, 8177−8183.

(8) Hu, S., Zhang, S., Hu, Z., Xing, Z., and Zhang, X. (2007) Detection of multiple proteins on one spot by laser ablation inductively coupled plasma mass spectrometry and application to immuno- microarray with element-tagged antibodies. *Anal. Chem. 79*, 923−929.

(9) Waentig, L., Techritz, S., Jakubowski, N., and Roos, P. H. (2013) A multi-parametric microarray for protein profiling: simultaneous analysis of 8 different cytochromes via differentially element tagged antibodies and laser ablation ICP-MS. *Analyst (Cambridge, U.K.) 138*, 6309−6315.

(10) Bandura, D. R., Baranov, V. I., Ornatsky, O. I., Antonov, A., Kinach, R., Lou, X., Pavlov, S., Vorobiev, S., Dick, J. E., and Tanner, S. D. (2009) Mass cytometry: technique for real time single cell multitarget immunoassay based on inductively coupled plasma time-of-flight mass spectrometry. *Anal. Chem. 81*, 6813−6822.

(11) Bodenmiller, B., Zunder, E. R., Finck, R., Chen, T. J., Savig, E. S., Bruggner, R. V., Simonds, E. F., Bendall, S. C., Sachs, K., Krutzik, P. O., and Nolan, G. P. (2012) Multiplexed mass cytometry profiling of cellular states perturbed by small-molecule regulators. *Nat. Biotechnol. 30*, 858−867.

(12) Tanner, S., Baranov, V., Ornatsky, O., Bandura, D., and George, T. (2013) An introduction to mass cytometry: fundamentals and applications. *Cancer Immunol. Immunother. 62*, 955−965.

(13) Han, G., Zhang, S., Xing, Z., and Zhang, X. (2013) Absolute and relative quantification of multiplex DNA assays based on an elemental labeling strategy. *Angew. Chem., Int. Ed. 52*, 1466−1471.

(14) Luo, Y., Yan, X., Huang, Y., Wen, R., Li, Z., Yang, L., Yang, C. J., and Wang, Q. (2013) ICP-MS-based multiplex and ultrasensitive assay of viruses with lanthanide-coded biospecific tagging and amplification strategies. *Anal. Chem. 85*, 9428−9432.

(15) Brückner, K., Schwarz, K., Beck, S., and Linscheid, M. W. (2014) DNA quantification via ICP-MS using lanthanide-labeled probes and ligation-mediated amplification. *Anal. Chem. 86*, 585−591.

(16) Lou, X., Zhang, G., Herrera, I., Kinach, R., Ornatsky, O., Baranov, V., Nitz, M., and Winnik, M. A. (2007) Polymer-based elemental tags for sensitive bioassays. *Angew. Chem., Int. Ed. 46*, 6111−6114.

(17) Majonis, D., Herrera, I., Ornatsky, O., Schulze, M., Lou, X., Soleimani, M., Nitz, M., and Winnik, M. A. (2010) Synthesis of a functional metal-chelating polymer and steps toward quantitative mass cytometry bioassays. *Anal. Chem. 82*, 8961−8969.

(18) Majonis, D., Ornatsky, O., Kinach, R., and Winnik, M. A. (2011) Curious results with palladium- and platinum-carrying polymers in mass cytometry bioassays and an unexpected application as a dead cell stain. *Biomacromolecules 12*, 3997−4010.

(19) Illy, N., Majonis, D., Herrera, I., Ornatsky, O., and Winnik, M. A. (2012) Metal-chelating polymers by anionic ring-opening polymerization and their use in quantitative mass cytometry. *Biomacromolecules 13*, 2359−2369.

(20) de Bang, T. C., Pedas, P., Schjoerring, J. K., Jensen, P. E., and Husted, S. (2013) Multiplexed quantification of plant thylakoid

proteins on Western blots using lanthanide-labeled antibodies and laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). *Anal. Chem. 85*, 5047−5054.
(21) Waentig, L., Jakubowski, N., Hardt, S., Scheler, C., Roos, P. H., and Linscheid, M. W. (2012) Comparison of different chelates for lanthanide labeling of antibodies and application in a Western blot immunoassay combined with detection by laser ablation (LA-)ICP-MS. *J. Anal. At. Spectrom. 27*, 1311−1320.
(22) De Leon-Rodriguez, L. M., and Kovacs, Z. (2008) The synthesis and chelation chemistry of DOTA-peptide conjugates. *Bioconjugate Chem. 19*, 391−402 and references therein..
(23) Lewis, M. R., Jia, F., Gallazzi, F., Wang, Y., Zhang, J., Shenoy, N., Lever, S. Z., and Hannink, M. (2002) Radiometal-labeled peptide-PNA conjugates for targeting bcl-2 expression: preparation, characterization, and in vitro mRNA binding. *Bioconjugate Chem. 13*, 1176−1180.
(24) De Leon-Rodriguez, L. M., Kovacs, Z., Dieckmann, G. R., and Sherry, A. D. (2004) Solid-phase synthesis of DOTA-peptides. *Chemistry 10*, 1149−1155.
(25) Boros, E., Polasek, M., Zhang, Z., and Caravan, P. (2012) Gd(DOTAla): a single amino acid Gd-complex as a modular tool for high relaxivity MR contrast agent development. *J. Am. Chem. Soc. 134*, 19858−19868.
(26) Overoye-Chan, K., Koerner, S., Looby, R. J., Kolodziej, A. F., Zech, S. G., Deng, Q., Chasse, J. M., McMurry, T. J., and Caravan, P. (2008) EP-2104R: a fibrin-specific gadolinium-based MRI contrast agent for detection of thrombus. *J. Am. Chem. Soc. 130*, 6025−6039.
(27) De Silva, C. R., Vagner, J., Lynch, R., Gillies, R. J., and Hruby, V. J. (2010) Optimization of time-resolved fluorescence assay for detection of europium−tetraazacyclododecyltetraacetic acid-labeled ligand−receptor interactions. *Anal. Biochem. 398*, 15−23.
(28) Merciny, E., Desreux, J. F., and Fuger, J. (1986) Séparation chromatographique des lanthanides en deux groupes basée sur des différences dans la cinétique de décomposition des complexes lanthanides-macrocycles. *Anal. Chim. Acta 189*, 301−311.
(29) Brücher, E., Laurenczy, G., and Makra, Z. S. (1987) Studies on the kinetics of formation and dissociation of the cerium(III)-DOTA complex. *Inorg. Chim. Acta 139*, 141−142.
(30) Wang, X., Jin, T., Comblin, V., Lopez-Mut, A., Merciny, E., and Desreux, J. F. (1992) A kinetic investigation of the lanthanide DOTA chelates. Stability and rates of formation and of dissociation of a macrocyclic gadolinium(III) polyaza polycarboxylic MRI contrast agent. *Inorg. Chem. 31*, 1095−1099.
(31) Cacheris, W. P., Nickle, S. K., and Sherry, A. D. (1987) Thermodynamic study of lanthanide complexes of 1,4,7-triazacyclononane-N,N′,N″-triacetic acid and 1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tetraacetic acid. *Inorg. Chem. 26*, 958−960.
(32) Tóth, É., and Brücher, E. (1994) Stability constants of the lanthanide(III)-1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tetraacetate complexes. *Inorg. Chim. Acta 221*, 165−167.
(33) Wu, S. L., and Horrocks, W. D. (1997) Direct determination of stability constants of lanthanide ion chelates by laser-excited europium(III) luminescence spectroscopy: application to cyclic and acyclic aminocarboxylate complexes. *J. Chem. Soc., Dalton Trans.*, 1497−1502.
(34) Rajagopal, A., Scherer, A., Homyk, A., and Kartalov, E. (2013) Supercolor coding methods for large-scale multiplexing of biochemical assays. *Anal. Chem. 85*, 7629−7636.
(35) Urdea, M. S., Warner, B. D., Running, J. A., Stempien, M., Clyne, J., and Horn, T. (1988) A comparison of non-radioisotopic hybridization assay methods using fluorescent, chemiluminescent and enzyme labeled synthetic oligodeoxyribonucleotide probes. *Nucleic Acids Res. 16* (11), 4937−4956.
(36) De Leon-Rodriguez, L. M., Ortiz, A., Weiner, A. L., Zhang, S., Kovacs, Z., Kodadek, T., and Sherry, A. D. (2002) Magnetic resonance imaging detects a specific peptide-protein binding event. *J. Am. Chem. Soc. 124*, 3514−3515.
(37) Yoo, B., Sheth, V. R., and Pagel, M. D. (2009) An amine-derivatized, DOTA-loaded polymeric support for Fmoc solid phase peptide synthesis. *Tetrahedron Lett. 50*, 4459−4462.
(38) Pazos, E., Torrecilla, D., Vazquez, L. M., Castedo, L., Mascarenas, J. L., Vidal, A., and Vazquez, M. E. (2008) Cyclin A probes by means of intermolecular sensitization of terbium-chelating peptides. *J. Am. Chem. Soc. 130*, 9652−9653.
(39) Becker, C. F. W., Clayton, D., Shapovalov, G., Lester, H. A., and Kochendoerfer, G. G. (2004) On-resin assembly of a linkerless lanthanide(III)-based luminescence label and its application to the total synthesis of site-specifically labeled mechanosensitive channels. *Bioconjugate Chem. 15*, 1118−1124.