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Novel approach for labeling of biopolymers with DOTA complexes using *in situ* click chemistry for quantification

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ABSTRACT

In this work, we present a two-step labeling approach for the efficient tagging with lanthanide-containing complexes. For this purpose, derivatization of the cysteine residues with an alkyne group acting as linker was done before the DOTA complex was introduced using *in situ* click chemistry. The characterization of this new methodology is presented including the optimization of the labeling process, demonstration of the quantitative capabilities using both electrospray ionization mass spectrometry (ESI-MS) and inductively coupled plasma mass spectrometry (ICP-MS) detection, and study of the fragmentation behavior of the labeled peptides by collision-induced dissociation (CID) for identification purposes. The results show that, in terms of labeling efficiency, this new methodology improves previously developed DOTA-based label strategies, such as MeCAT-maleimide (metal-coded affinity tag, MeCAT-Mal) and MeCAT-iodoacetamide (MeCAT-IA) reagents. The goal of reducing the steric hindrance caused by the voluminous DOTA complex was fulfilled allowing both, quantification and identification of labeled biopolymers.

1. Introduction

The introduction of mass spectrometry (MS) into life sciences and biotechnology has provided a powerful tool for the identification of proteins. However, qualitative analysis is only part of the proteomics analysis, and the need for quantitative proteomics is becoming more frequent for applications concerning the status of biological systems [1]. Different quantification techniques have been developed to address this question using stable isotope labeling and molecular MS detection [2].

Some examples of these techniques are stable isotope labeling by amino acids in cell culture (SILAC) [3], and isotope-coded affinity tags (ICAT) [4]. The latter, labels cysteine groups through alkylation using reagents containing iodoacetamide groups. On the other hand, isobaric tag for relative and absolute quantitation (iTRAQ) [5] and isotope-coded protein label (ICPL) [6] are strategies which label the sample through specific modification of the primary amines. These quantification techniques work by comparing two samples each labeled with a heavy and a light version of the same reagent. The absolute quantification of the sample requires more elaborate strategies where standards of the target species must usually be employed for calibration [7,8]. At this point, the structure-dependent response of molecular MS techniques is an issue for signal calibration.

Metal-coded affinity tag (MeCAT) was introduced by Ahrends et al. [9] as a new labeling reagent for absolute and sensitive quantification of proteins and peptides. This reagent allows tracking of labeled species by ICP-MS, while taking advantage of its high sensitivity, multiplexing capabilities, and structure-independent response for quantification purposes [1,2]. MeCAT tags were devised to label peptides and proteins with lanthanide ions which are loaded into DOTA complexes (1,4,7,10-tetraazacyclododecane-N, N′,N″,N‴-tetraacetic acid) containing maleimide residues which are cysteine reactive. New generations of this reagent based on cysteine reactive iodoacetamide group (MeCAT-IA) or alternative DOTA labeling using primary amine reactive N-Hydroxysuccinimide derivative [10,11] or isothiocyanatobenzyl derivative [12] have been also employed to label proteins and peptides. Furthermore, consecutive labeling of several amino acid residues to enhance the sensitivity of the detection or track different biological states has been done [13]. The analytical robustness of the DOTA-based labeling and its suitability for proteomics using MS/MS analysis [14,15] was demonstrated [10,16–19]. However, due to the size of the DOTA complexes and spatial hindrance in peptides and proteins, some of the active sites are inaccessible [10]. To deal with this problem, we are aiming to reduce the steric hindrance and enable higher number of active sites to be accessible by a two-step labeling strategy involving *in situ* click chemistry. Since its discovery in 2002, the copper catalyzed azide-alkyne cycloaddition (CuAAC) reaction [20], which nowadays

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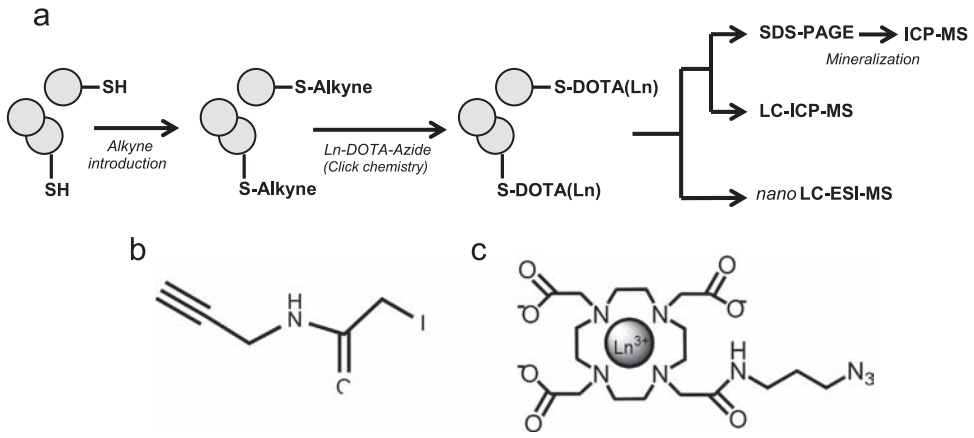

Fig. 1. (a) Workflow for quantification of peptides and proteins using the proposed two-step labeling procedure. (b) Structure of 2-iodo-N-(prop-2-yn-yl)acetamide alkyne (INA). (c) Ln–DOTA–azide complex.

is widely recognized as click chemistry, has been frequently employed in different fields [21–24]. This attractive reaction has been optimized [25] and applied successfully to biological samples, including living cells [26].

In the present work, a new two-step labeling procedure was studied to improve the labeling efficiency of biopolymers with lanthanide-containing DOTA complexes (Fig. 1a). First, the thiol group of cysteine was modified with a small residue containing a terminal alkyne (Fig. 1b), therefore avoiding the direct introduction of bulky residue causing steric hindrance. This small group was devised as a spacer that can offer more accessibility to the second label. After that, without any washing step, the metal harboring DOTA-Azide complex (Fig. 1c) was introduced using click chemistry. In this second step, the high yield of the click reaction allows to reach high labeling efficiency. The general features of this strategy, including the optimized experimental conditions for high labeling efficiency, the effect of different loading metals, the CID fragmentation of the labeled species for identification, and the suitability for peptide and protein quantification are presented.

2. Materials and methods

2.1. Labeling of standard peptide

Firstly, the reduction process of standard peptide WWCNDGR (Schafer-N, Copenhagen, Denmark) was performed with three-fold excess of *tris*(2-carboxyethyl) phosphine (TCEP, Sigma Aldrich, Germany) per disulfide bond in 100 mM Triethylammonium bicarbonate buffer (TEAB buffer, pH 8.5, Fluka, Switzerland), incubated at 50 °C for 1 h. Then, three-fold excess of 2-iodo-N-(prop-2-yn-yl)acetamide alkyne (INA) per thiol group was added to the reduced peptide. The reaction was conducted under gentle shaking at room temperature in darkness overnight.

Once the alkyne was introduced into the peptide, the Ln-DOTA-azide labeling was attempted using *in situ* click chemistry. The alkyne modified peptide (30 μM) and eight-fold excess of Ln-DOTA-azide per thiol group were mixed. 15-fold excess of *tris* [(1-hydroxypropyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (THPTA) per thiol group was premixed with cupric sulfate ($CuSO_4$, Sigma Aldrich, Germany) in the ratio of 9:1 before addition to the mixture. Then, aminoguanidine (Sigma Aldrich, Germany) was added according to 20 fold-excess the $CuSO_4$. At the end, sodium ascorbate (Sigma Aldrich, Germany) was added to the reaction (50 fold-excess compared to $CuSO_4$) to ignite the reaction by reducing Cu(II) to Cu(I). This click chemistry reaction was incubated in darkness for 1 h at 30 °C in Ar atmosphere under sonication (Elmasonic S15H ultrasonic bath, Germany).

2.2. Tryptic digestion of standard BSA

Bovine serum albumin (BSA, Sigma Aldrich, Germany) was prepared in 50 mM ammonium bicarbonate (pH 8.0, Carl Roth, Karlsruhe, Germany) at 10 mg mL^{-1}. Denaturation procedure was conducted at 50 °C for 30 min by adding 10% acetonitrile. Three-fold excess of TCEP per disulfide bond was added to BSA solution, and then incubated for 1 h at 50 °C for reduction.

After reduction, trypsin (Sequencing Grade Modified, Promega, Germany) was added into the solution in the ratio of 1:100 (trypsin:protein). After the first incubation step at 37 °C for 4 h, a second aliquot of trypsin was added, and then incubated overnight at 37 °C.

2.3. Labeling of digested and intact standard BSA

Alkyne labeling of the digested BSA was performed in 100 mM TEAB buffer at pH 8.5 with 20% acetone at room temperature in darkness with gentle shaking overnight. To obtain quantitative labeling, six-fold excess of INA per thiol was added to the reaction mixture.

In the click chemistry stage, alkyne labeled tryptic digest of BSA and eight-fold excess of Ln-DOTA-azide per thiol group were mixed. THPTA was premixed with $CuSO_4$ in the ratio of 9:1 in TEAB buffer before addition to the mixture. The amount of $CuSO_4$ in the solution was not lower than 9% molar concentration of Ln-DOTA-azide. Then, aminoguanidine was added according to 20 fold-excess the $CuSO_4$. Finally, sodium ascorbate was added to the reaction (50 fold-excess compared to $CuSO_4$). The reaction solution was incubated for 1 h at 30 °C in Ar atmosphere under sonication.

The optimized labeling procedure for tryptic digest of BSA was also applied to label intact BSA. However, in the click chemistry reaction, denaturation with 6 M urea was necessary for highly efficient labeling of the intact protein.

2.4. SDS-PAGE to monitor intact protein labeling

SDS-PAGE was used to evaluate the labeling efficiency of intact BSA. The metal-labeled proteins were separated in 10% (w/v) polyacrylamide gels and visualized staining with colloidal coomassie G-250 solution (Carl Roth, Germany) after gel fixation with 40% methanol (MeOH, J.T. Baker, Netherland) and 13.5% formalin (37%, Carl Roth, Germany). Roti-Mark Standard (Karl Roth, Germany) was used for molecular weight calibration.

2.5. nanoLC(RP)–ESI-FT-ICR-MS analysis of labeled peptides

Standard labeled peptides were analyzed by *nano*LC Fourier transform ion cyclotron resonance-mass spectrometry (*nano*LC (RP)–ESI-FT-ICR-MS, Thermo Fisher Scientific, Bremen, Germany). A *nano*LC system (Agilent 1100 series, Germany) equipped with a Zorbax 300 SB-C18 (150 mm × 75 μm, Agilent, Germany) analytical column and a Zorbax 300 SB-C18 (0.3 mm × 5 mm, Agilent, Germany) trap column for desalting and pre-concentration purposes were used. The mobile phases were: phase A: 5% acetonitrile (v/v, ACN, J.T. Baker, Netherlands), 0.1% formic acid (v/v, FA, Fluka, Switzerland); phase B: 99.9% ACN (v/v), 0.1% FA (v/v). For identification of the separated peptides, the *nano*LC was coupled to ESI-FT-ICR-MS using a nanomate ESI interface working at 1.7 kV (Advion, USA). The analysis was conducted in positive mode using a transfer capillary temperature of 200 °C. The *nano*LC(RP)–ESI-FT-ICR-MS signal was used to calculate the labeling efficiency as the chromatographic peak area percent of the labeled peptide in comparison with the sum of the labeled and unlabeled peptide areas. Chromatographic peak areas of different forms of the selected peptides were determined by their extracted ion chromatogram (EIC) with a mass tolerance of 10 ppm.

2.6. ICP-MS analysis of labeled proteins and peptides

Prior to ICP-MS (Element XR, Thermo Fisher Scientific, Bremen, Germany) measurements by direct infusion sample introduction, the gel bands were excised and mineralized overnight at 95 °C in a closed vial using a mixture of 150 μL nitric acid (HNO_3, ultraquality, Carl Roth, Germany) and 60 μL H_2O_2 (30%, Sigma Aldrich, Germany). Before measurement, 3 μL solution was diluted to 500 μL solution with 3.5% (v/v) HNO_3. ^{141}Pr at 5 ppb was used as internal standard to correct possible instrumental drifts during the measurement.

2.7. LC(SCX)–ICP-MS analysis of labeled proteins and peptides

Peptides and proteins labeled with Ln-MeCAT-Click were analyzed using Agilent 1200 series system (Agilent, Germany) equipped with a BioBasic strong cation exchange analytical column (SCX, 5 μm, 150 mm × 1 mm, Thermo Fisher Scientific, Germany) coupled to the ICP-MS configured with a MCN-600 desolvating membrane nebulizer (CETAC, USA). The mobile phases were: phase A, 10% MeOH (v/v), 0.1% FA (v/v); phase B, 10% MeOH (v/v), 200 mM ammonium acetate (Carl Roth, Germany), pH 3 (adjusted with FA). The LC elution gradient is shown in Table S1.

3. Results and discussion

3.1. Optimization of the two-step labeling strategy

The labeling conditions were optimized starting with the alkyne spacer introduction. In the first labeling step, pH was a critical parameter and was optimized independently for peptide and protein. According to the results shown in Fig. S1, the quantitative labeling of the standard peptide WWCNDGR with the INA was achieved at pH values not lower than 8.2. Similar result was observed for tryptic digest of BSA. In the second labeling step introducing Ln-DOTA-azide by *in situ* click chemistry, pH value lower than 8.5 did not lead to complete labeling (data not shown). For this reason, pH values no lower than 8.5 are recommended for cysteine derivatization with the alkyne reagent.

The click chemistry reaction was tackled following Hong's approach [26], where THPTA is used to intercept reactive oxygen species (ROS) and protect Cu(I) from oxidation. Based on the results obtained after the optimization of the THPTA amounts (Fig. S2a), nine equivalents of THPTA relative to $CuSO_4$ were required in this reaction. Lower excess of this ligand failed to lead to quantitative labeling, while higher excess could suppress the reaction. At least eight-fold excess of Ln-DOTA-azide with respect to the thiol groups are required to quantitatively label the peptide under the described conditions (Fig. S2b).

To maintain the pH of the mixture, Ln-DOTA-azide was dissolved in 0.1 M TEAB buffer (pH 8.5) before addition. Otherwise, the addition of the acidic Ln-DOTA-azide decreases the pH of the solution and causes incomplete labeling.

The special use of sonication must be emphasized in this point. The sonication-assisted reaction led to quantitative labeling of alkyne modified BSA with the Ln-DOTA-Azide after 1 h at 30 °C. The result agreed with Tu et al. [27], who used sonication to promote click chemistry between small molecules in a meso-flow reactor synthesis system. However, in the present study, it was utilized for the first time to enhance the click chemistry efficiency and achieve quantitative labeling of biomolecules at low temperature in short time. Under Ar atmosphere, the degassing effect of sonication could help to get rid of O_2 in the solution which could destabilize Cu(I) [25]. In Table S2, it can be clearly observed the different labeling efficiency of alkyne-modified tryptic peptides from BSA when the sonication bath was used or when the sample was only shaken.

Without sonication, complete labeling of alkyne-modified tryptic peptides from BSA can be also achieved through click chemistry in 2 h at 50 °C. However, at this high temperature, some problems arise for intact protein where urea was present, and carbamylation of N-termini and lysine residues could take place [28]. Denaturation with urea was necessary for high efficient labeling on intact proteins in the click chemistry reaction. The absence of urea led to precipitation of the protein in this step, and the intensity on the SDS-PAGE gel was noticeably reduced (Fig. S3).

Furthermore, reaction temperatures higher than 30 °C caused degradation of the proteins in the presence of sodium ascorbate and Cu(II) [29,30]. Therefore, sonication-assisted procedure is suitable for intact protein labeling since it allows complete labeling at lower reaction temperature (30 °C) which is a requirement for subsequent quantification.

3.2. Quantitative analysis of standard peptide WWCNDGR labeled with Ln-MeCAT-Click

The labeling efficiency was estimated using the *nano*LC(RP)–ESI-FT-ICR-MS signal from the labeled and unlabeled peptide. Unlabeled and alkyne labeled peptides were not detected after the second step (data not shown), therefore, both labeling steps were considered quantitative. CID fragmentation experiments were conducted to check the compatibility of the two-step labeling strategy with conventional MS/MS based identification. The fragmentation behavior of Ho-MeCAT-Click labeled peptide WWCNDGR with CID fragmentation technique is shown in Fig. 2. Typical b- and y-series ions were formed and detected after CID fragmentation, allowing unambiguous identification.

The same standard peptide was labeled with MeCAT-Click reagents loaded with different lanthanides showing chromatographic co-elution, which is a requirement for multiplex quantification. As it is shown in Fig. 3a and b, peptide WWCNDGR labeled with three different metals co-elute both in reversed phase chromatography and strong cation exchange chromatography.

^{165}Ho, ^{169}Tm, and ^{175}Lu were selected due to their high isotope abundance (100% abundance for ^{165}Ho and ^{169}Tm, and 97.41% for ^{175}Lu) and the significant mass differences between the monoisotopic peaks (4Da between Ho and Tm, and 6Da between Tm and Lu). In this way, negligible overlapping of the isotope patterns was expected.

The same biomolecule labeled with different lanthanides presented the expected mass shift enabling the relative quantification by ESI-MS. In addition, ICP-MS determination was also possible comparing the signal of the lanthanides loaded into the DOTA-labels. In general, the relative abundances of the three different mixtures determined by these two complementary instruments were in good agreement with the spiked relative amounts (Fig. 3c, d) with relative measurement errors generally lower than ±4% and relative standard deviations around ±5% for all the mixtures. As in the case of other MeCAT reagents [11,17,31], the labeled species can also be quantified with molecular and elemental MS techniques allowing both structural information and high sensitive quantification.

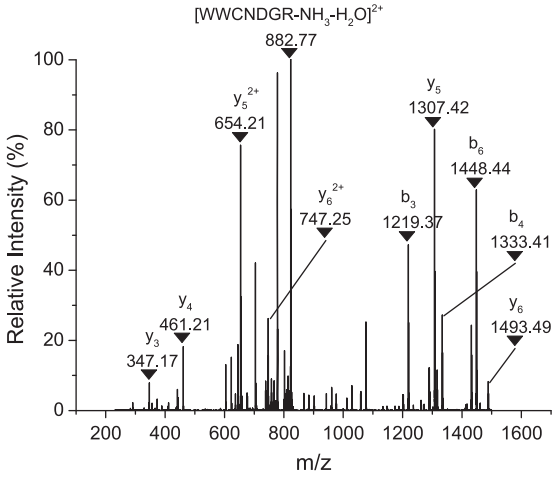

Fig. 2. CID fragmentation spectrum (ESI-FT-ICR-MS) of the standard peptide WWCNDGR labeled with Ho-MeCAT-Click.

3.3. Quantitative analysis of digested BSA labeled with Ln-MeCAT-Click

The two-step labeling approach was also applied to more complex samples such as digested BSA. For tryptic digest of BSA, among 25 cysteine-containing peptides, 23 were found labeled with Ho-MeCAT-Click after click chemistry reaction (Table 1). The peptides, DVCK and CCAADDK were totally absent and were not found, neither unlabeled nor labeled, which was also observed in previous studies with other MeCAT labels [10,13]. Poor ionization in the ESI source or insufficient retention of these peptides in the chromatographic system could explain the absence. The peptide ECCHGDLLECADDR can be found fully or incompletely labeled with only one lanthanide atom out of three possible reactive sites. It is worth mentioning, that all the other peptides with two adjacent cysteine residues were completely labeled, and ECCHGDLLECADDR was the only exception. Both, the alkyne labeling and Ho-MeCAT-Click labeling significantly improved the

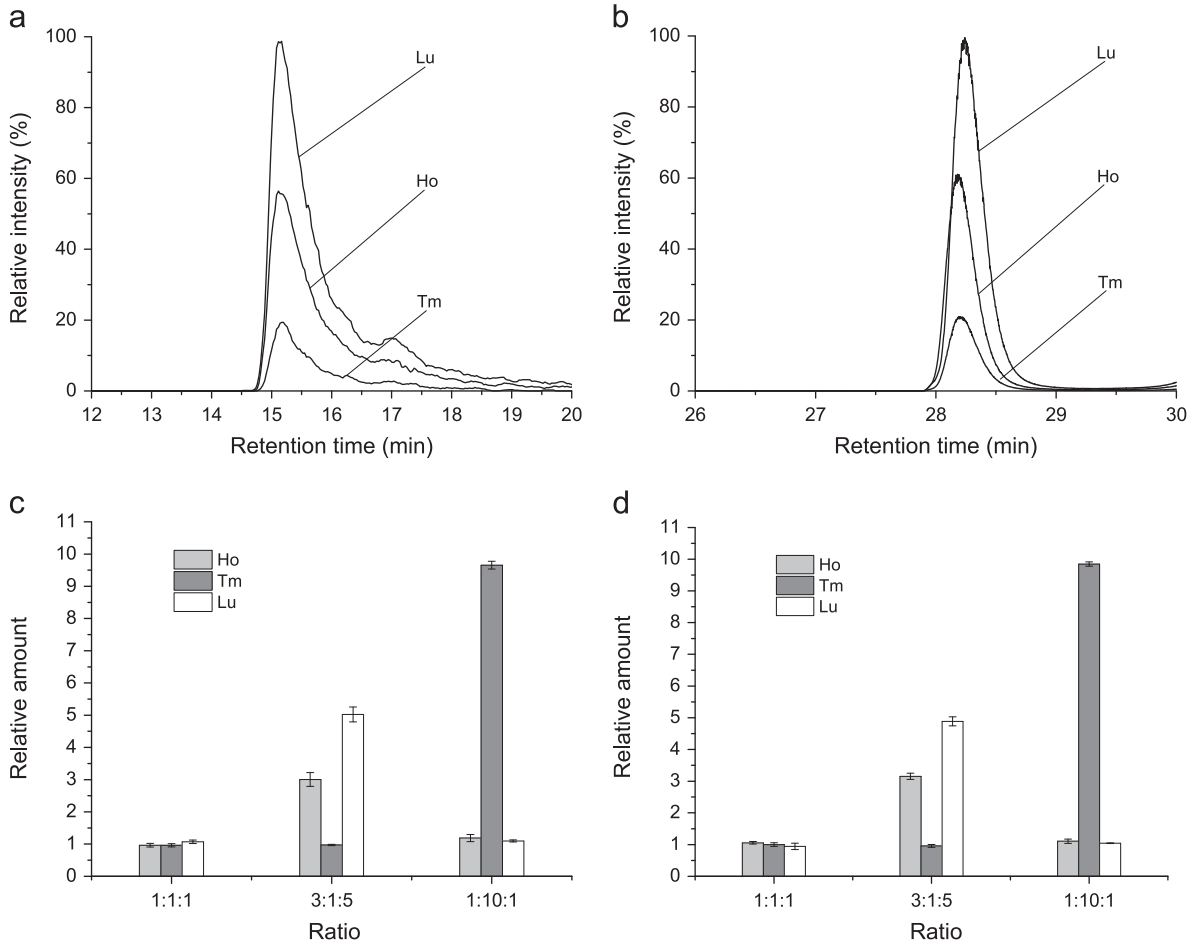

Fig. 3. (a) *nano*LC(RP)–ESI-FT-ICR-MS elution of Ln-MeCAT-Click labeled peptide WWCNDGR (Ho:Tm:Lu ratio 3:1:5). (b) LC(SCX)–ICP-MS elution of Ln-MeCAT-Click labeled peptide WWCNDGR (Ho:Tm:Lu ratio 3:1:5). (c) Relative quantification by *nano*LC(RP)–ESI-FT-ICR-MS of the standard peptide WWCNDGR labeled with different Ln-MeCAT-Click labels and mixed in different proportions (Ho:Tm:Lu ratios 1:1:1, 3:1:5 and 1:10:1). (d) Relative quantification by LC(SCX)–ICP-MS of the standard peptide WWCNDGR labeled with different Ln-MeCAT-Click labels and mixed in different proportions (Ho:Tm:Lu ratios 1:1:1, 3:1:5 and 1:10:1). Uncertainties correspond to standard deviation from three replicates, $n=3$.

Table 1
Analysis by *nano*LC(RP)–ESI-FT-ICR-MS of a tryptic digest of BSA labeled with different MeCAT labels. In brackets it is shown the number of completely labeled cysteines with respect to the total number of cysteines per peptide.

Peptide sequence	MeCAT labeling approach		
	MeCAT-Mal [10]	MeCAT-IA [10]	MeCAT-Click
^{45}GLVLIAFSQYLQQCPFDEHV K^{65}	+(1/1)[a]	+(1/1)	+(1/1)
^{76}TCVADESHAGCEK^{88}		+(2/2)	+(2/2)
	+(1; 2/2)		
^{89}SLHTLFGDELCK^{100}	+(1/1)	+(1/1)	+(1/1)
^{106}ETYGDMADCCEK^{117}		+(2/2)	+(2/2)
	+(2/2)		
^{123}NECFLSHK^{130}	+(1/1)	+(1/1)	+(1/1)
^{139}LKPDPNTLCDEFK^{151}	+(1/1)	+(1/1)	+(1/1)
^{184}YNGVFQECCQAEDK^{197}		+(2/2)	+(2/2)
	+(2/2)		
^{198}GACLLPK^{204}	+(1/1)	+(1/1)	+(1/1)
^{223}CASIQK^{228}	+(1/1)	+(1/1)	+(1/1)
^{267}ECCHGDLLECADDR^{280}		+(3/3)	+(1,3/3)
	+(1/3)		
^{286}YICDNQDTISSK^{297}	+(1/1)	+(1/1)	+(1/1)
^{300}ECCDKPLLEK^{309}		+(2/2)	+(2/2)
	+(2/2)		
^{310}SHCIAEVEK^{318}	+(1/1)	+(1/1)	+(1/1)
^{337}DVCK^{340}	+(1/1)	+(1/1)	–
^{375}EYEATLEECCAK^{386}		+(2/2)	+(2/2)
	+(2/2)		
^{387}DDPHACYSTVFDK^{399}	+(1/1)	+(1/1)	+(1/1)
^{413}QNCDQFEK^{420}	+(1/1)	+(1/1)	+(1/1)
^{460}CCTKPESER^{468}		+(1; 2/2)	+(2/2)
	+(1; 2/2)		
^{469}MPCTEDYLSLILNR^{482}	+(1/1)	+(1/1)	+(1/1)
^{483}LCVLHEK^{489}	+(1/1)	+(1/1)	+(1/1)
^{499}CCTESLVNR^{507}		+(1; 2/2)	+(2/2)
	+(2/2)		
^{508}RPCFSALTPDETYVPK^{523}	+(1/1)	+(1/1)	+(1/1)
^{529}LFTFHADICTLPDTEK^{544}	+(1/1)	–	+(1/1)
^{581}CCAADDK^{587}		+(1; 2/2)	–
	–		
^{588}EACFAVEGPK^{597}	+(1/1)	+(1/1)	+(1/1)

[a] +: detected, −: not detected.

sequence coverage, which may be due to enhanced ionization efficiency of the peptides in the ESI or to improved chromatographic separation hence improving the detection by molecule-based MS (Table S2).

Therefore, around 96% of detected cysteine-containing peptides were completely labeled, while according to the literature less than 90% of them were found completely labeled in the best case when other DOTA-based labeling strategies were used [10]. The lower steric hindrance utilizing this click-chemistry-based approach after the introduction of an alkyne spacer enhanced the labeling efficiency, offering advantages over other MeCAT labeling strategies such as MeCAT-Mal and MeCAT-IA [10] (Table 1). Therefore, this new two-step approach opens the door for promising applications which are currently running on much more complicated samples where the complexity and difficulty to label are usually main issues.

To demonstrate that this methodology can be employed to quantify peptide mixtures in a conventional proteomics workflow, the tryptic digest of BSA were separately labeled with ^{165}Ho, ^{169}Tm and ^{175}Lu. Afterwards, the different metal-coded BSA samples were mixed together in different ratios, and analyzed by LC (SCX)–ICP-MS and *nano*LC(RP)–ESI-FT-ICR-MS.

For elemental MS measurements, we used the same strategy as employed for standard peptides. A short LC elution gradient (Table S1) devised to first elute the excess Ln-DOTA-Azide, and then elute the labeled peptides all together, was used. The relative quantification was done by calculating the whole area of the two neighboring peaks containing all the Ln-MeCAT-Click labeled peptides (retention time between 15 and 19 min, Fig. 4a). The very first major peak (retention time 3.1 min) is the excess Ln–DOTA–azide. The calculations for all the tested Ho:Tm:Lu ratios are presented in Fig. 4b, where experimental values were very close to the expected ones.

For molecular MS measurements, two peptides, DDPHACYSTVFD and SLHTLFGDELCK were chosen for peptide relative quantification. Since the relative amount of protein in the mixtures can be determined from the corresponding peptide ratios, protein quantification was also possible. Satisfactory results for relative quantification were also obtained, and are shown in Fig. 4c, and d, with relative errors (*ca.* ±5%) and relative standard deviations (*ca.* ±5%) slightly higher than for the same mixtures analyzed by LC(SCX)–ICP-MS.

3.4. Quantitative analysis of intact BSA labeled with Ln-MeCAT-Click

SDS-PAGE was used to evaluate the labeling efficiency on the protein level. Both alkyne and Ln-DOTA-Azide labeling were quantitatively performed using the optimized labeling conditions (Fig. S3). In addition, the labeling efficiency of this two-step labeling strategy on intact BSA was also confirmed by *nano*LC (RP)–ESI-FT-ICR-MS after tryptic digestion of the labeled protein. Similar results to those from labeling the tryptic digest of BSA were achieved. Furthermore, no unlabeled cysteine-containing peptides could be detected (Table S2).

Relative quantification on protein level was performed by labeling BSA with different Ln-MeCAT-Click labels (loaded with ^{165}Ho, ^{169}Tm or ^{175}Lu). Different mixtures of the differentially labeled BSA were separated by SDS-PAGE (Fig. 5a). Using this technique, excess of the

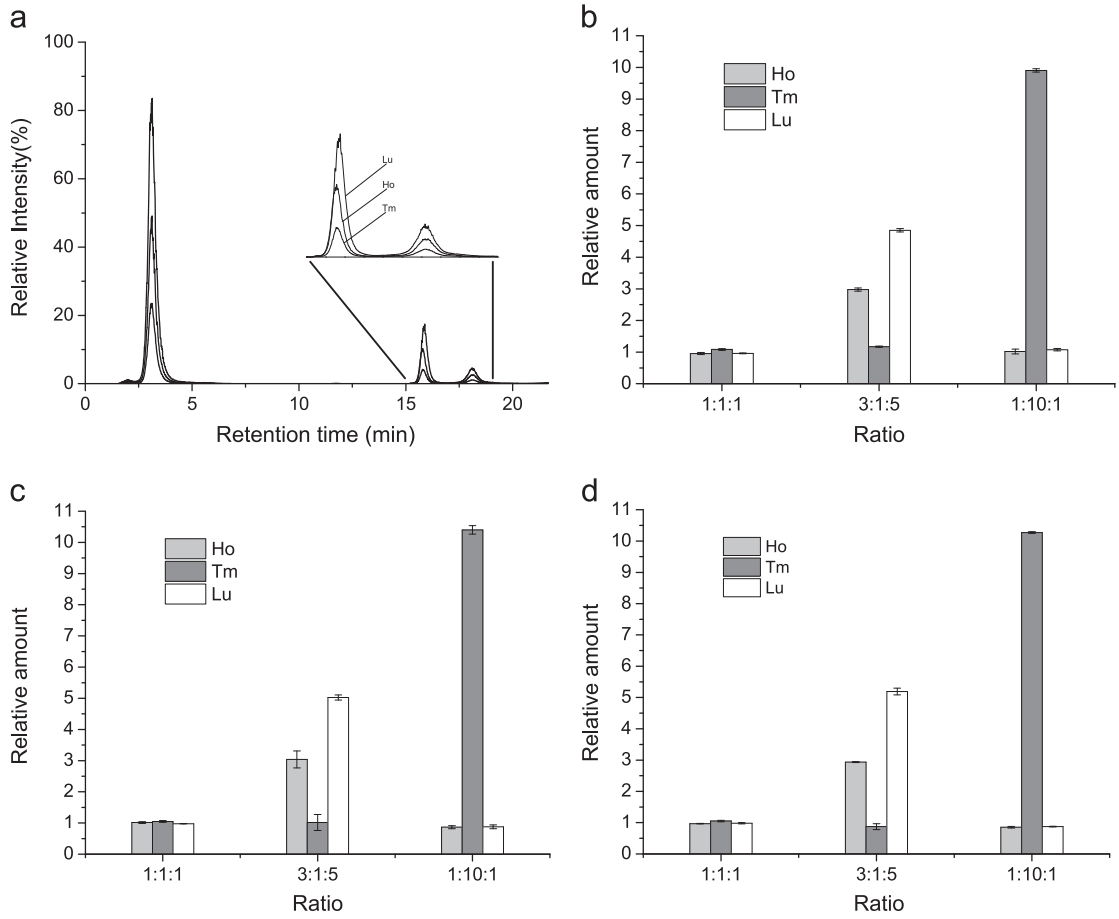

Fig. 4. (a) LC(SCX)–ICP-MS elution of digested BSA labeled with Ln-MeCAT-Click labels (Ho:Tm:Lu ratio 3:1:5). (b) Relative quantification by LC(SCX)–ICP-MS of digested BSA labeled with Ln-MeCAT-Click labels which were mixed in different proportions (Ho:Tm:Lu ratios 1:1:1, 3:1:5 and 1:10:1). Relative quantification by *nano*LC(RP)–ESI-FT-ICR-MS of (c) DDPHACYSTVFDK and (d) SLHTLFGDELCK, peptides from tryptic digest of BSA labeled with different Ln-MeCAT-Click labels and mixed in different proportions (Ho:Tm:Lu ratios 1:1:1, 3:1:5 and 1:10:1). Uncertainties correspond to standard deviation from three replicates, $n=3$.

Ln-DOTA-Azide was efficiently separated from the labeled species, and consequently lanthanide background in subsequent analysis reduced. After excision and mineralization of the gel bands, the solution was analyzed by direct infusion ICP-MS. The protein ratios were determined from the relative signal intensities of the corresponding lanthanides. Once more the results match very well with the expected values as can be seen in Fig. 5b.

We also employed LC(SCX)–ICP-MS to quantify mixtures of the intact BSA labeled with Ln-MeCAT-Click click tags containing different lanthanides. Using the same elution gradient that used for peptides before, the excess of Ln-DOTA-Azide was eluted before the elution of the labeled proteins (Table S1). In Fig. 5c, a chromatogram can be observed, where two peaks correspond to different isoforms of the labeled BSA (retention time between 26 and 28 min). Both isoforms were completely labeled with Ln-MeCAT-Click as confirmed by *nano*LC(RP)–ESI-FT-ICR-MS (data not shown). The first peak corresponds to the excess Ln-DOTA-Azide (retention time 3.1 min). In this case, the relative quantification was done by calculating the sum of the areas of both peaks. Once again the ratios fit well with the spiked relative protein amounts, with similar errors and standard deviations as those observed in the previous measurements (Fig. 5d).

4. Conclusion

In this study, a new approach is presented for labeling thiol residues in peptides and proteins following a two-step based strategy including *in situ* click chemistry reaction for quantification. Advantages in terms of labeling efficiency have been demonstrated in comparison with previous DOTA based labels, such as MeCAT-Mal and MeCAT-IA.

The voluminous DOTA complexes may create steric hindrance that inhibits the derivatization of possible binding sites. The introduction of an easy-to-react alkyne spacer in the cysteine residues result in better accessibility of the bulky Ln-DOTA-Azide in the subsequent labeling step. Improving the labeling efficiency, even at the protein level, is an important feature for application to complex samples. The absence of washing steps or clean-up during the labeling process, avoids loss of sample or contaminations, and brings robustness to the approach.

The developed strategy employs MeCAT-based reagents, and is a promising tool for the sensitive quantification of proteins using ICP-MS as detector. Moreover, different approaches for the analysis of the labeled samples have been applied such as, liquid chromatography separations or gel electrophoresis followed by mineralization and direct infusion ICP-MS, both with intact and digested proteins.

The employed labels are compatible with ESI-MS techniques, which allows identification of labeled peptides and proteins. High sensitive determination of heteroatoms by ICP-MS combined with identification capability of ESI-MS can improve quantitative and qualitative information of biopolymers.

The developed approach creates a solid base for further application to biological samples where the efficiency in the labeling is an important issue.

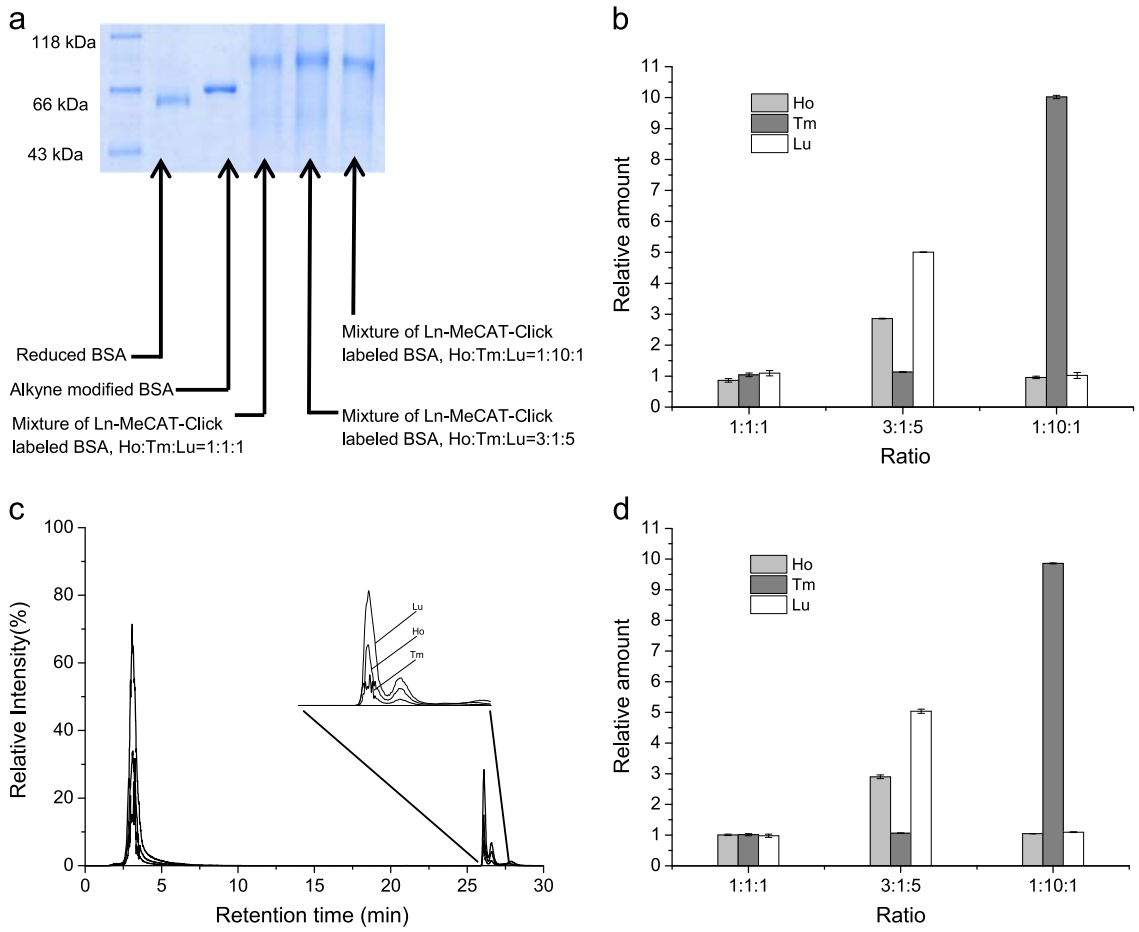

Fig. 5. (a) SDS-PAGE and (b) relative quantification by direct infusion ICP-MS after gel band mineralization of intact BSA labeled with different Ln-MeCAT-Click labels which were mixed in different proportions (Ho:Tm:Lu ratios 1:1:1, 3:1:5 and 1:10:1). (c) LC(SCX)–ICP-MS elution of intact BSA labeled with different Ln-MeCAT-Click labels mixed in the ratio of 3:1:5 Ho:Tm:Lu. (d) Relative quantification by LC(SCX)–ICP-MS of intact BSA labeled with different Ln-MeCAT-Click labels and mixed in different proportions (Ho:Tm:Lu 1:1:1, 3:1:5 and 1:10:1). Uncertainties correspond to standard deviation from three replicates, $n=3$.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.11.049.

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