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Synthesis and characterization of a new MeCAT reagent containing a photocleavable linker for labeling of proteins and peptides in mass spectrometric analyses

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ABSTRACT

The quantification of proteins and peptides becomes more important besides mere identification in modern life sciences. Therefore, we have developed a new reagent that adds to the known metall-coded affinity tagging strategy employed in molecular and elemental mass spectrometry containing a photocleavable linker. A synthesis route was developed that provides the new reagent in good yields. The stability of the synthesized reagents was assessed under different temperature and illumination conditions. Labeling reactions were carried out at peptide and protein level, while also the fragmentation behavior of labeled peptides was assessed. In additional experiments, the photocleavability of the new reagent was examined. Upon irradiation with ultraviolet light, the photoproducts were liberated and could be used for quantification of labeled peptides.

1. Introduction

Besides the sequencing of genetic information from DNA or RNA, proteomics has become a pivotal and substantial part in the quest to understand biological processes. Most proteomics approaches target the identification of proteins and their post-translational modifications. Both can be directly achieved by the use of mass spectrometric (MS) methods. However, the mere identification of gene products and their modifications only delivers limited information on the dynamics of a proteome at different developmental stages and under certain environmental factors. Quantification of proteins has thus become an important tool to understand biochemical functions and to unravel underlying mechanisms in living cells [1]. In quantitative proteomics, two main approaches exist: labeling free and stable isotope labeling [2]. In label free methods, the quantitative information is gained by comparison of the chromatographic peak area or the MS signal height in survey scans in relation to an added standard [3]. The standards often contain heavy isotopes like ^{13}C or ^{15}N while being structurally identical to the target, as ideally they have to be indistinguishable from the target in their physico-chemical properties (e.g. retention time in HPLC). Although label free methods that rely on spectral counting have become more and more popular, especially for large data sets, these appear to yield rather semi-quantitative results in comparison to proper quantitative methods [4]. Isotope labeling methods like SILAC, ICPL, ITRAQ or TMT label peptides or proteins directly with heavy isotopes and interfere relative quantification by comparison of the signals heights in MS scans [5,6]. The advantages of these are that signal differences (e.g. due to different ionization or detector response) are neglectable and whole proteomes can be directly assessed. However, only relative data are obtained and only the identical protein species can be compared in different cell states.

Absolute quantification delivers absolute concentrations or amounts and thus always contains the relative information for comparison of different proteins. Furthermore, in absolute quantifications results are comparable even if they were obtained by different methods. We had developed metal-coded affinity tagging (MeCAT) for absolute quantification. MeCAT utilizes lanthanides (and other heavy metals) that are contained in a chemical tag [7–9]. MeCAT tags directly enable the use of inductively coupled plasma (ICP)-MS to determine absolute quantities [10,11]. For relative quantification of different samples this quantitative information can be easily converted into relative data. Furthermore, relative quantification can be achieved by using electrospray (ESI)- or matrix-assisted laser desorption/ionization (MALDI)-MS, comparable to isotope-labeling methods. While the MeCAT reagents have initially been based on maleinimide coupling to cysteine residues [12], MeCAT has been further improved over the years. Additions to the MeCAT reagent family have also been introduced, including sulfenic acid and amino group specificity or utilization of click chemistry for labeling [1,13–17].

Here, we introduce a new MeCAT reagent employing a photocleavable linker that can be used to specifically label cysteine residues

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and to liberate a reporter from labeled peptides and proteins upon ultraviolet (UV) irradiation. There are several compounds that can be used as photocleavable building block, including phenacyl ester [18,19] or coumarin derivatives [20,21]. The best-characterized light-sensitive linkers consist of ortho-nitrobenzyl derivatives, whose absorption is in the near UV region [22–26].

2. Materials and Methods

2.1. Synthesis and stability of the photocleavable reagents

The first steps of the synthesis were carried out according to [27]. MeCAT-IA was obtained by the synthetic route described in detail previously [28]. Due to the possible photocleavage of the linker by daylight, all steps from the nitration step onward were performed in the dark. Further details of the synthesis are described in the Supporting information. For stability assessment, three aqueous 20 µM solutions of AMBA-Ln-MeCAT-IA **[8]** containing Tm were stored for 1 h in incubators of 37 °C, 50 °C and 95 °C each, in the dark. The resulting samples were directly subjected to high-performance liquid chromatography (HPLC)-MS. Daylight stability tests were performed with 20 µM solutions of AMBA-Ln-MeCAT-IA **[8]** (Ln=Tm) in Eppendorf tubes at room temperature (RT). Samples were allowed to sit on the window sill (closed window) for 1, 3 and 13 days and subjected to HPLC-MS analyses afterwards.

2.2. Labeling of peptides and proteins

For peptide labeling, a 200 µM aqueous solution of α-lactalbumin (LALBA, Sigma-Aldrich) was reduced with 50 mM of Tris(2-carboxyethyl)phosphine (TCEP, 98%, Sigma-Aldrich) at 50 °C for 30 min. Subsequently, the protein solution was diluted with ammonium bicarbonate solution (50 mM final concentration) to 50 µM and trypsin (modified sequencing grade, Promega) was added to a final molar ratio of LALBA:trypsin of 100:1. This mixture was incubated for 4 h at 37 °C, when the identical amount of trypsin was added again, followed by incubation at 37 °C overnight. The resulting peptide mixture was labeled in a solution containing 75 mM borate buffer (pH 8.2) and a threefold molar excess per cysteine residue of AMBA-Ln-MeCAT-IA **[8]**. This solution was incubated at RT overnight in the dark. For protein labeling, an aqueous 200 µM LALBA solution was reduced by addition of a TCEP (50 mM final concentration) and incubated for 30 min at 50 °C. 2.5 µL of this reduced LALBA solution were labeled by addition of 0.6 µL of a 20 mM (3 equ. excess per cysteine residue) AMBA-Ln-MeCAT-IA **[8]** solution, 28.2 µL sodium borate buffer (133 mM; pH 8.2), 18 mg urea, 10 µL acetone und 8.7 µL water. The resulting mixture was incubated at RT overnight in the dark. Peptide and protein labeling mixtures were directly used for HPLS-MS analyses.

2.3. Photolysis

For examination of photocleavability, completely labeled peptide mixtures of LALBA were irradiated by a conventional handheld laboratory UV lamp (12 W, Carl Roth) in Eppendorf tubes with open lid. The UV irradiation (λ = 365 nm) resulted in a photoinduced reaction.

2.4. HPLC-MS and fragmentation

HPLC-MS analyses were carried out on a LTQ XL or a LTQ FTICR Ultra (both Thermo Fisher Scientific). An Agilent 1200 HPLC-system was coupled via ESI to the MSs, while peptide separation was performed on a C_{18}-column (Luna C18(2), 150 × 1.0 mm, 5 µm, Phenomenex). A C_3-cartridge (Zorbax 300SB-C3, 2.1 × 12.5 mm, 5 µm, Agilent) was employed for protein analyses. An injection volume of 5 µL and a gradient of A:water/acetonitrile/formic acid 94.9/5.0/0.1 (v/v/v) and B:acetonitrile/formic acid 99.9/0.1 (v/v) was used. The gradient program for the C_{18} column was as follows: 0 min 90% A, 20 min 50% A, 22 min 5% A, 28 min 5% A, 30 min 90% A, 35 min 90% A, while the flow rate was 40 µL min^{-1}. For the C_3 column, the gradient was 0 min 90% A, 20 min 70% A, 25 min 5% A, 28 min 5% A, 30 min 90% A, 35 min 90% A with a flow rate of 15 µL min^{-1}. For automated data dependent fragmentation, the five most abundant peptide signals in FTICR spectra were subjected to collision induced (CID) fragmentation and detection in the ion trap with an isolation width of Δm/z = 3 and an activation energy of 35%.

2.5. Automated database searches

Automated database searches were accomplished using Proteome Discoverer 1.4.1.12 (Thermo Fisher Scientific) with the Sequest algorithm. The AMBA-Ln-MeCAT-IA was implemented as dynamic artificial modification with the mass differences of the added label ($C_{37}H_{45}N_8O_{13}$SLn) per cysteine residue upon labeling: Tb – 1000.2075, Ho – 1006.2124, Tm – 1010.2163, Lu – 1016.2229.

3. Results and discussion

3.1. Synthesis of the reagent

To fulfill the requirements for a photocleavable reagent and to limit molecular size/weight of it, we decided to implement an aromatic ortho-nitro aromatic functionality into the reagent. Synthesis of the lanthanide containing photocleavable MeCAT reagents **[8]** has been carried out by the route shown in Fig. 1 (for details see Supporting information).

As starting compound the commercially available AMBA **[1]** was used. This was converted with trifluoroacetic anhydride to obtain **[2]**. In the next step, **[2]** was nitrated by fuming nitric acid to yield 3-Nitro-4-(trifluoroacetylaminomethyl)benzoic acid **[3]**. Subsequently, the amino function of **[3]** was deprotected by alkaline hydrolysis and re-protected with a *tert*-butyloxycarbonyl (*t*-boc) group to result in **[4]**. The next step in the synthesis was coupling with Cystamine dihydrochloride utilizing the coupling reagents 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and Hydroxybenzotriazole (HOBt) to **[5]** that contains two aromatic ortho-nitro groups per molecule. Finally, **[5]** was deprotected to **[6]**. This intermediate step was stored at −20 °C in the dark and served as the starting compound for the remaining steps of the synthesis. The disulfide bond of **[6]** was reduced with an excess of Tris(2-carboxyethyl)phosphine (TCEP) and subjected to coupling with the previously described MECAT-IA [16,28] to yield **[7]**, which was purified by preparative HPLC. Under the given conditions (see Supporting information), **[7]** eluted between 35 and 39 min. This retention time was independent from the lanthanide employed. Fractions of **[7]** were collected, pooled from multiple injections and freeze-dried. In the final synthesis step, the iodoacetamide function was introduced, which is responsible for the specific binding to the peptide and protein thiol residues. For that, **[7]** was dissolved in a $NaHCO_3$-solution and treated with an excess of N-succinimidyl iodoacetate (SIA) to result in the photocleavable AMBA-Ln-MeCAT-IA reagent **[8]** that was also purified by preparative HPLC.

3.2. Reagent stability tests

Once the AMBA-Ln-MeCAT-IA **[8]** had been obtained, we performed a number of stability test to ensure that the reagent could be used routinely without exciding precautions or limitations. General storability and usability was assessed by subjecting the reagent to 37 °C, 50 °C and 95 °C followed by HPLC-MS analyses. At 37 °C and 50 °C, no reduction in peak areas of the reagent was detected and as a result no degradation or lysis was observed. At 95 °C, a reduction of the peak area of the reagent in corresponding HPLC-MS chromatograms was apparent. Hence, the reagent **[8]** proved to be not stable at 95 °C.