CILAT – a new reagent for quantitative proteomics†

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We report the development of a new reagent, called CILAT (Cleavable Isobaric Labeled Affinity Tag), for quantitative proteomics, which represents an improvement over current ICAT (Isotope-Coded Affinity Tag) and iTRAQ (Isobaric Tags for Relative and Absolute Quantitation) methods.

Since the introduction of the ICAT method,[1] tandem mass spectrometry (MS/MS) has become an indispensable tool for pair-wise comparison of protein abundance due to its high throughput and high sensitivity.[2] It allows quick identification and accurate quantitation of differentially expressed proteins from two or more biological samples, making it a powerful technique that can be applied to a wide range of areas, including systematic mapping of protein phosphorylation,[3] biomarker discovery for various diseases,[4] and inspection of drug-response.[5] The prototype ICAT reagents consist of a biotin affinity tag, a linker coded with either deuterium or hydrogen atoms as the base for quantitation, and a thiol-capture reactive group that selectively labels cysteine, a rare amino acid in proteins.[1] As a result, cysteine-containing peptides can be enriched from proteolytic peptide mixtures upon treatment with ICAT reagents, which reduces the complexity of biological samples and simplifies subsequent MS/MS assays. Each pair of peptides with a unique mass-shift is then identified and measured for their relative amount. Recently, the ICAT–MS/MS approach has been further advanced by the development of new generations of ICAT reagents with improved properties, such as the incorporation of cleavable linkers to remove the biotin tag that results in superior MS/MS spectral quality[6,7] and the introduction of $^{13}C/^{12}C$ nuclei that eliminates chromatographic shift caused by the deuterated tags.[8] Nevertheless, labeling the same peptides with mass-different ICAT tags can also adversely increase the complexity of MS analysis, which is made worse by the fact that many peptides can be labeled with multiple tags. Furthermore, cysteine-free proteins are undetectable by the ICAT–MS/MS method. To overcome these limitations, the iTRAQ method has been developed to provide an alternative for protein quantitation.[9] Peptides derivatized with a multiplex set of iTRAQ reagents are indistinguishable in MS, but can generate distinct strong low-mass signature ions upon fragmentation in MS/MS that can be measured quantitatively. Therefore, these isobaric tags can simplify MS complexity over mass-difference labeling because there is no splitting of precursor signal. Sensitivity of detection is also enhanced in that all ions of the same peptides labeled with the different iTRAQ reagents contribute to the same signal peak. However, unlike ICAT, iTRAQ molecules are amine-reactive, hence they uniformly label any tryptic peptides and require other means to reduce biological complexity.

Here, we report the development of the CILAT approach that would take advantage of benefits offered by both affinity enrichment and isobaric tagging. As illustrated in Fig. 1a, besides a biotin affinity tag and an acid-labile linker, these CILAT reagents have an isobaric tag consisting of a reporter group and a carbonyl balance group where either position 1 or 2 is encoded with a ^{13}C nucleus, and a thiol group as the capture agent for tyrosine-containing peptides. This design was based on a well-known reaction: tyrosine can be oxidized to ortho-quinone by tyrosinase, a copper-containing enzyme that is involved in melanin synthesis.[10] Ortho-quinone is a very active intermediate that undergoes rapid and efficient Michael addition to form covalent

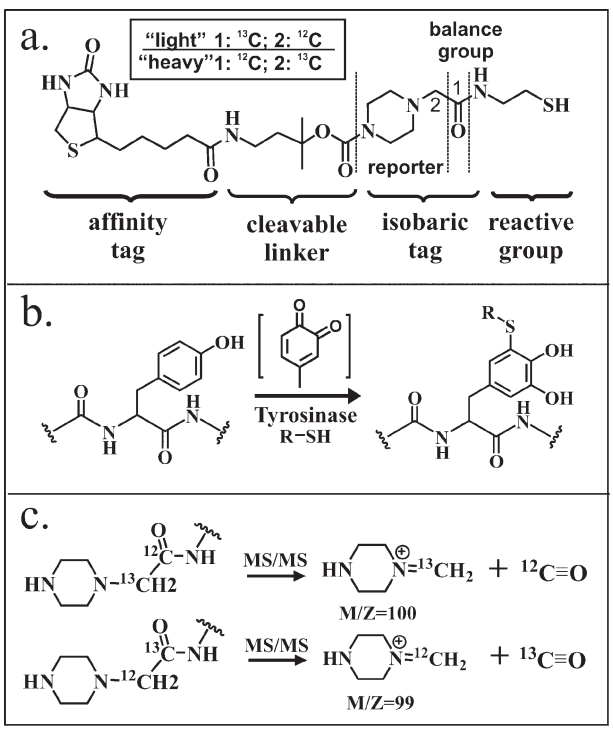

Fig. 1 (a) Structure of the CILAT reagents. These compounds consist of a biotin affinity tag, an acid-cleavable linker, an isobaric tag comprised of a reporter group and a carbonyl balance group, as well as a reactive group to capture tyrosine. The "light" tag is encoded with 1-^{13}C and 2-^{12}C. The "heavy" tag is encoded with 1-^{12}C and 2-^{13}C. (b) Tyrosine can form a tyrosine–thiol adduct *via* an ortho-quinone intermediate catalyzed by tyrosinase. (c) The "light" and "heavy" isobaric tags produce two signature peaks at m/z = 99 and m/z = 100 respectively upon MS/MS fragmentation.

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adducts with thiol-containing molecules (Fig. 1b).[11] We recently demonstrated that other naturally occurring amino acids, except cysteine which is usually alkylated in a proteomic assay, did not interfere with this reaction.[12] Tyrosinase also exhibited promiscuous activity, and was potentially able to react with every tyrosine-containing peptide with a different sequence.[12] When the compound encoded with a 1-^{13}C atom ("light" tag) was incubated with a tryptic peptide mixture, tyrosine-containing peptides would be labeled and enriched with immobilized monoavidin. Subsequently, the bulky biotin on these peptides would be removed with acid treatment. Upon the fragmentation of a precursor ion in MS/MS, the amide bond in the tag would then be broken apart, followed by the spontaneous neutral loss of the carbonyl group to yield a positively-charged *N*-methylpiperazine fragment with *m*/*z* equivalent to 99 (Fig. 1c). Other fragments should still be isobaric and could be used to deduce the sequence of the parent peptide. The same procedure using the other reagent encoded with a 2-^{13}C atom ("heavy" tag) would produce the reporter ion with *m*/*z* equivalent to 100. The relative intensity of these two signature peaks would then be measured to quantitate protein expression levels from two different states.

To demonstrate this idea, we first developed a simple strategy to prepare the CILAT reagents on solid support beads (see supplementary material). This solid phase synthesis allowed us to prepare the CILAT compounds on a peptide synthesizer automatically with a modified program, rendering it easy for scale-up. We then digested two equal amounts of purified bovine serum albumin (BSA) with trypsin and treated the resulting peptide mixtures with the "light" and "heavy" tags respectively. After combining two samples together, we enriched the biotinylated peptides with monoavidin–agarose and analyzed them with an ABI 4700 MALDI-TOF/TOF mass spectrometer. The tyrosine-bearing peptides with C-terminal arginine dominated the mass spectrum in that these peptides ionize better than those ending with C-terminal lysine (Fig. 2a). The two strongest peaks were then selected for MS/MS assay to determine their sequences with CID (collision induced dissociation) off, which were identified as the singly charged ions of the peptides H-MPCTEDYLSLILNR-OH (Fig. 2c, *m*/*z* = 1942.9) and H-LGEYGFQNALIVR-OH (see supporting material, *m*/*z* = 1697.8) respectively. This mass increment relative to the unmodified peptides was due to the addition of the tag on tyrosine (Fig. 2b), which increased the mass of the parent ion by 218, and the alkylation on cysteine, which increased the mass by 57. As expected, both peptides were found to be a 1 : 1 mixture based on the ratio of two strong signature peaks at *m*/*z* = 99 and *m*/*z* = 100 when CID was turned on (Fig. 2d and supporting material), indicating the success of our approach.

Because tyrosine is a more abundant amino acid in proteome (3.35%) than cysteine (0.99%),[13] the CILAT method should significantly increase peptide coverage relative to ICAT and still allow us to reduce biological complexity with affinity enrichment. Tryptic peptides with C-terminal arginine, which usually exhibit a strong signal in MS assay, would have better chances to contain tyrosine than cysteine residues, thus being enriched and providing higher signal/noise ratio for protein identification. The same peptides derivatized with either isobaric tag would be indistinguishable in MS, both simplifying the mass spectra and enhancing

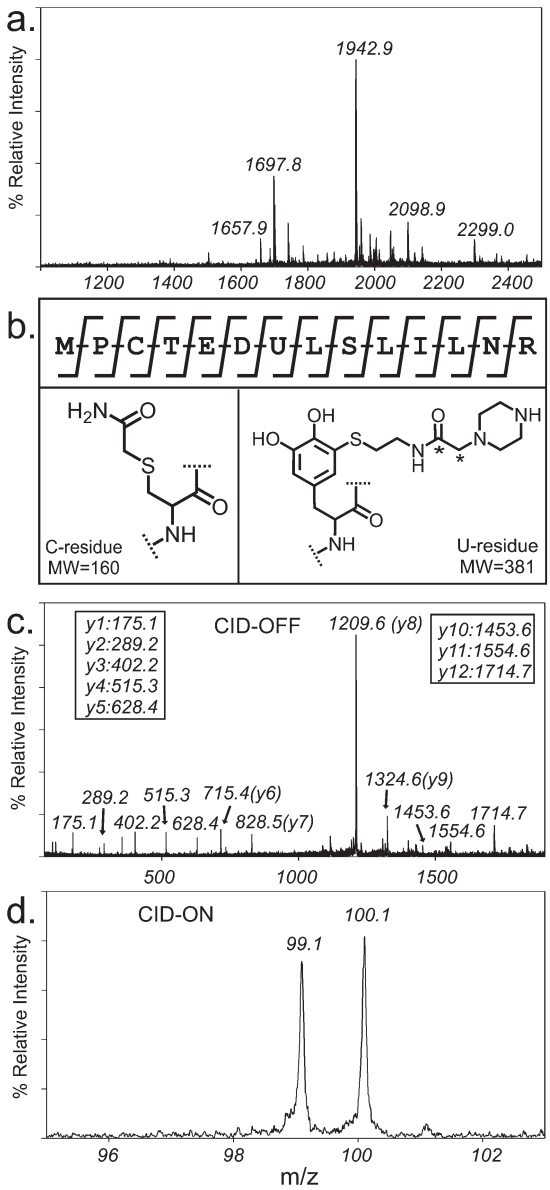

Fig. 2 (a) MS spectrum of the tryptic BSA peptides after CILAT labeling and affinity purification with immobilized avidin. (b) Structure of the peptide corresponding to the peak 1942.9 in MS. Cysteine is alkylated by reacting with bromoacetamide. U is the tyrosine–thiol adduct which is modified as described in the paper. Either position labeled with an asterisk (*) is encoded with a ^{13}C atom. (c) MS/MS spectrum of the precursor ion at 1942.9 with CID off. Y-series peaks are labeled. (d) Signature peaks of the precursor ion at 1942.9 after MS/MS fragmentation with CID on.

the sensitivity of detection. In addition, the module solid phase synthesis of the CILAT molecules would make it easy to exchange each component, such as the replacement of the biotin with a fluorous tag as a different route for affinity purification.[14] Finally, these CILAT reagents could also be applied to quantitative phosphoproteome research by coupling with the β-elimination of phosphoserine and phosphothreonine residues.[3] The ease and robustness of this technique will ensure its wide use in both academic and biomedical applications.

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