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Absolute Quantitation of Proteins by Coulometric Mass Spectrometry

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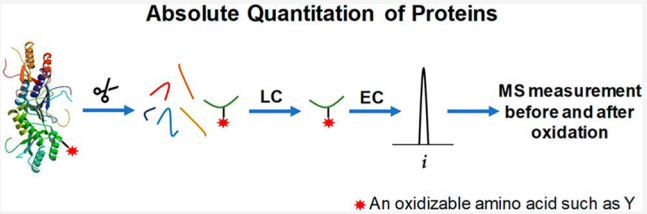
ABSTRACT: Accurate quantification is essential in the fields of proteomics, clinical assay, and biomarker discovery. Popular methods for absolute protein quantitation by mass spectrometry (MS) involve the digestion of target protein and employ isotope-labeled peptide internal standards to quantify chosen surrogate peptides. Although these methods have gained success, syntheses of isotope-labeled peptides are time-consuming and costly. To eliminate the need for using standards or calibration curves, herein we present a coulometric mass spectrometric (CMS) approach for absolute protein quantitation, based on the electrochemical oxidation of a surrogate peptide combined with mass spectrometric measurement of the oxidation yield. To demonstrate the utility of this method, several proteins were analyzed such as model proteins β-casein, and apomyoglobin as well as circadian clock protein KaiB isolated from *Escherichia coli*. In our experiment, tyrosine-containing peptides were selected as surrogate peptides for quantitation, considering the oxidizable nature of tyrosine. Our data showed that the results for surrogate peptide quantity measured by our method and by traditional isotope dilution method are in excellent agreement, with the discrepancy of 0.3−3%, validating our CMS method for absolute quantitation. Furthermore, therapeutic monoclonal antibody (mAb) could be quantified by our method as well. Due to the high specificity and sensitivity of MS and no need to use isotope-labeled peptide standards, our CMS method would be of high value for the absolute proteomic quantification.



Accurate protein quantitation is a fundamental requirement in a multitude of biological research areas.[1−8] An accurate measurement of absolute protein amount in a sample can be performed by quantitative proteomics approaches such as selected reaction monitoring (SRM) or high-resolution mass spectrometry (HRMS) in combination with isotope-labeled standards.[9] The method requires a known concentration of internal standard, typically doped in the tryptic digest of the target protein, which is labeled with isotopically heavy atoms to mimic native surrogate peptide formed by proteolysis. The sample mixture containing the isotope-labeled peptide standard and the surrogate peptide is then analyzed by LC-MS. Similar to the relative quantitation using isotope-labeled standards, peptides of equal chemistry coelute and are analyzed by MS simultaneously. Using a predetermined calibration curve, the ion abundances of the surrogate peptide and the isotope-labeled standard are compared to calculate the absolute quantity of the target surrogate peptide, which represents the target protein content. Thus, absolute quantitation is a targeted quantitative proteomics technique that exhibits robust efficacy and has been increasingly utilized for a wide variety of quantitative proteomics studies.[10−14]

Nevertheless, it has some associated drawbacks. First, isotope-labeled standard peptides are synthesized de novo, which takes time and their syntheses are very costly. The synthesized peptide standards contain more or less some impurities (typically up to 5% impurity). Second, the ionization efficiencies for the heavy and light isotope-labeled peptides, although very close, are not exactly the same, which may contribute to quantitation error.[15,16] Third, the heavy isotope-labeled and the light peptides sometimes cannot have exactly the same chromatographic elution time (e.g., for deuterium labeled peptides), making their precise comparison difficult.[17] Fourth, there is an upper limit for multiplexing analysis using isotope-labeling approach, as simultaneous comparison of too many peptides with different isotope-labeling in one MS spectrum can be difficult.[18,19] More importantly, any existing background peak that overlaps with the isotope-labeled peptide standard peak would prevent the use of this method. Therefore, new strategies for improved MS-based protein quantitation are still in need.

In this study, we present the development of a conceptually new approach of using electrochemistry (EC)-assisted mass spectrometry for absolute quantitation of proteins. The striking

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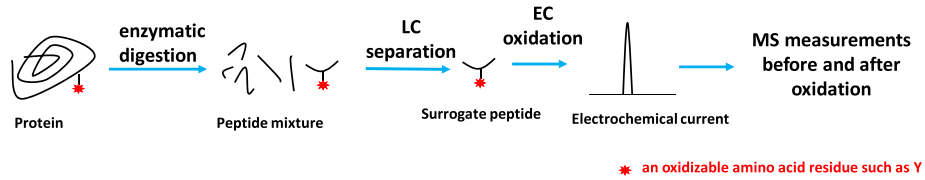
Scheme 1. Schematic Showing Our Approach for Absolute Quantitation of Protein



feature of our method is that our method does not need the use of any standard or isotope-labeled peptides or any standard curves, for absolute quantitation.

As illustrated in Scheme 1, in our approach, a target protein is first digested into peptides. By using chromatographic separation, a peptide containing electrochemically active residue (e.g., tyrosine), can be separated out and chosen as a surrogate signature peptide and then introduced for oxidation in an electrochemical flow cell, followed with MS detection. Electric current is generated and recorded during electrochemical oxidation. Based on the Faraday's Law, Q, the total electricity, can be calculated by integrating the Faradaic current over time. Q is related to the moles of the peptide that has been oxidized, as shown in the equation of $Q = nzF$ (n, z and F denote the moles of the oxidized peptide, the number of electrons lost from oxidizing every peptide molecule, and the Faraday constant of 9.65×10^4 C/mol, respectively). Thus, n is equal to Q/zF. Upon oxidation, ion intensity of the surrogate peptide decreases. The relative reduction of the peptide ion peak area in the recorded MS data before and after the electrochemical oxidation reflects the oxidation yield (denoted as Δi). Thus, the total amount of the surrogate peptide can be calculated as the quotient of the amount of the oxidized peptide n and the oxidation yield Δi (i.e., $Q/(zF\Delta i)$).

Recently we have shown that this CMS method can be used for accurately quantifying small molecules[20−22] and tyrosine- or cysteine-containing peptides including phosphopeptides.[23] In this study, the method is applied to absolute protein quantitation, for the first time, as protein can be digested into peptides and stoichiometrically one protein molecule typically produces one peptide molecule in theory (antibody can be an exception). By using our approach, we successfully quantified the absolute amounts of β-casein, apomyoglobin, and KaiB proteins expressed from *E. coli*. The peptide amount measured by CMS is in excellent agreement with traditional isotope dilution method (≤3% difference) and also can reflect the amount of the corresponding protein. Our measurement error for quantitation of these proteins, defined as the difference between the moles of surrogate peptides and the moles of the corresponding proteins (determined from either the known weight or the Bradford Assay), ranges from −11.1 ∼ -12.8%. The negative error indicates there is likely a slight sample loss during the tryptic digestion of proteins to peptides. IgG2 antibody can also be quantified by CMS method. The relatively large quantitation error of −26.4% is likely due to the difficulty in digesting this large protein.

EXPERIENTIAL SECTION

Chemicals. Apomyoglobin from horse skeletal muscle (protein sequencing grade) and β-casein from bovine milk (bioultra grade) were bought from Sigma-Aldrich (St. Louis, MO). The monoclonal antibody drug Vectibix (panitumumab, IgG2) was purchased from Amgen (Thousand Oaks, CA). Peptides GGYR, DRVY and Arg^8-vasotocin (sequence: CYIQNCPRG, two cysteines are connected with one disulfide bridge) in HPLC grade were obtained from Genscript Biotech (Piscataway, NJ). Stable isotope-labeled peptide standards AVPYPQR^ (labeled at arginine, ^{13}C6, ^{15}N4, 95% purity) and VL^IGLDLLYGELQDSDDF (labeled at leucine, ^{13}C6, ^{15}N, 95% purity) were both purchased from New England Peptide (Gardner, MA). Trypsin (sequencing grade) was purchased from Promega (Madison, WI). Acetonitrile (ACN, HPLC grade) and acetone (ACS grade) were bought from Fisher Scientific (Fair Lawn, NJ). Formic acid (HPLC grade), urea (electrophoresis grade), ammonium bicarbonate (bioultra grade), dithiothreitol (DTT, bioultra grade), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, bioultra grade), and iodoacetamide (IAA, bioultra grade) were all bought from Sigma-Aldrich (St. Louis, MO). A Millipore Direct-Q5 purification system (Burlington, MA) was used to obtain purified water for sample preparation.

Protein Expression and Purification. The cloning and purification of KaiB were performed as described previously with minor modifications.[24,25] Basically, the open reading frame of KaiB from *Synechococcus elongatus* was amplified using polymerase chain reaction (PCR) and cloned into the pET-28b expression vector with a small ubiquitin-related modifier (SUMO) using NdeI and HindIII cloning sites. *Escherichia coli* BL21(DE3) was used for the overexpression of KaiB. Transformed *E. coli* culture was grown in 1 L LB at 37 °C until A_{600} reaches 0.7. The culture was induced with 1 mM isopropyl β-D-thiogalactopyranoside (Calbiochem, San Diego, CA) to overexpress recombinant KaiB. After 6 h, the cells were harvested and stored at −80 °C overnight. The cell pellets were resuspended in the buffer (500 mM NaCl, 20 mM Tris·HCl, 5 mM imidazole, and pH 7.0). The cell lysate was clarified by centrifugation at 20 000*g* for 60 min at 4 °C. The supernatant was filtered with 0.45 μm vacuum filter to remove small particles. The His-tagged KaiB was purified with Ni column and the anion-exchange chromatography was applied for further purification. Ulp1 protease was added to the eluent to cut the tag out. The His-tag was cleaved from KaiB after incubation at 4 °C overnight. To separate KaiB from His-tag, Ni column was used again, and another anion-exchange column was applied to complete the purification. The purity was checked by SDS/PAGE and dialyzed with the buffer (150 mM NaCl, 20 mM Tris·HCl, pH 8.0). The expressed KaiB protein was concentrated and determined at a concentration of 206 μM by the Bradford protein assay. The protein sample was then stored at −80 °C, prior to CMS quantitation.

Proteolytic Digestion. We dissolved 200 μg of β-casein from bovine milk in 50 mM ammonium bicarbonate (NH_4HCO_3, pH 7.4) followed by adding 50 μL of 0.2 μg/μL trypsin solution. The protein sample was incubated at 37 °C for overnight. The digested β-casein sample was diluted to

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the final concentration of 30 μM by adding mobile phase A (water with 0.1% formic acid).

We dissolved 100 μg of apomyoglobin from horse skeletal muscle in 50 mM ammonium bicarbonate (NH_4HCO_3, pH 7.4) followed by adding 50 μL of 0.2 μg/μL trypsin solution. The protein sample was incubated at 37 °C for overnight. The digested apomyoglobin sample was slightly diluted to the final concentration of 25 μM by adding mobile phase A (water with 0.1% formic acid).

The Vectibix IgG2 antibody was efficiently acetone precipitated and on-pellet digested by a reported procedure[26,27] with minor modifications. Briefly, the total concentration of the IgG2 was adjusted to 4 mg/mL, and 25 μL was precipitated by addition of three aliquots of cold acetone (50 μL each) while vortexing. The sample was incubated overnight at −20 °C and then centrifuged at 12 000g for 20 min. The supernatant was carefully removed and the pellet was air-dried at room temperature. Then a 50 μL of 0.08 μg/μL trypsin (in 50 mM Tris buffer, pH 8) was added to dissolve the pellet. After a brief vortexing, the sample solution was incubated in a water bath at 37 °C for 4 h. After that the sample was reduced with 5 mM TCEP at 95 °C for 5 min, and then alkylated with 10 mM IAA at 37 °C for 30 min in the dark. A second aliquot of trypsin (0.1 μg/μL, 40 μL) was added to the sample solution. The sample was incubated at 37 °C overnight to achieve complete digestion. The final volume of the digested solution was 100 μL, and the final concentration of digested antibody was 3.4 μM.

A 100 μg amount of KaiB protein was precipitated from the expressed protein sample solution using cold acetone at −20 °C overnight to remove salts and buffers. The protein pellet was obtained by centrifugation at 13 000g for 10 min. Then protein pellet was washed by cold acetone once and air-dried at room temperature. After that, the protein pellet was redissolved in 100 μL of 8 M urea. The protein was reduced by DTT at 37 °C for 30 min and alkylated by IAA at room temperature in the dark for 30 min. DTT was added again to quench extra amount of IAA. The sample was diluted by adding 100 mM NH_4HCO_3 to reduce urea concentration lower than 2 M. Four μg of trypsin (protease: protein = 1:25, w/w) was added into the protein sample solution and incubated at 37 °C for overnight. The digestion process was terminated by adding 1% formic acid (v/v). Peptides were then desalted by using C18 spin columns from G-Biosciences (St. Louis, MO). The desalted peptides were collected and diluted to the final concentration of 28 μM by adding mobile phase A (water with 0.1% formic acid) before LC/EC/MS analysis.

Instrumentation. As shown in Supporting Information (SI) Scheme S1, the experimental setup consisted of a ultraperformance liquid chromatography (UPLC, Waters, Milford, MA) coupled with an electrochemical thin-layer flow cell (BASi, West Lafayette, IN; cell dead volume: ca. 1 μL) and a high-resolution Orbitrap Q Exactive mass spectrometer (Thermo Scientific, San Jose, CA). The electrochemical cell used a glassy carbon disc (i.d., 6 mm, catalog# MF-1015) as the working electrode (WE). An Ag/AgCl (3 M NaCl) working electrode electrode was adopted as the reference electrode (RE, catalog# RE-6) and the cell stainless steel body served as a counter electrode (CE, catalog# MF-1092). A reversed phase column (BEH C18, 2.1 mm × 50 mm, 1.7 μm) was used for separation. A potential of +0.95 V or +1.05 V (vs Ag/AgCl) was applied to WE to trigger the oxidation of LC-separated peptides (the potential used in this study was chosen and optimized for achieving selective oxidation of tyrosine-containing peptides due to the relatively low oxidation potential of tyrosine residue compared to other residues[23]). A ROXY potentiostat (Antec BV, The Netherlands) was used to monitor and record the oxidation current. OriginPro 2018b was used to import and integrate the electric current peak to calculate the total electric charge of Q. The peptide flowing out of electrochemical cell was online analyzed using the Orbitrap mass spectrometer equipped with a heated electrospray ionization (HESI) source. The flow rate of sheath gas and the applied ionization voltage were 10 L/h and the +4 kV, respectively. The ion transfer inlet capillary temperature was kept at 250 °C. Mass spectra were acquired by Thermo Xcalibur (3.0.63). The working electrode were cleaned by polishing with alumina slurry after use.

For LC/MS analysis of peptide mixtures, the mobile phase flow rate was 200 μL/min. In a gradient elution, the mobile phase B (acetonitrile with 0.1% formic acid) increased from 5% to 8% in 3 min, and reached 15% in 1 min. Then, mobile phase B was reduced back to 10% in 1 min and kept at 10% for 4 min before returned to 5%. The concentrations of GGYR, DRVY, and Arg^8-vasotocin in the peptide mixture were 20 μM, 20 μM, and 25 μM, respectively. The injection volume was 3 μL per analysis.

For LC/MS setup of digested β-casein, apomyoglobin, and KaiB, the mobile phase flow rate was 200 μL/min. The mobile phase B (acetonitrile with 0.1% formic acid) increased from 5% to 40% in 10 min, and climbed to 70% from 10 to 15 min. Then, the mobile phase B went back to 5% in 1 min, and then remained at 5% for 4 min. The concentrations of digested β-casein, apomyoglobin, and KaiB were 30 μM, 25 μM, and 28 μM, respectively. The injection volume was 3 μL per analysis.

For LC/MS setup of digested antibody, the mobile phase flow rate was 200 μL/min. The mobile phase B (acetonitrile with 0.1% formic acid) increased from 5% to 20% in 5 min, and reached 30% from 5 to 25 min. Then, the mobile phase B climbed to 95% in 1 min and remained at 95% for 5 min. After that the mobile phase B returned to 5% in 1 min and finally isocratic at 5% for 5 min. The concentration of digested IgG2 antibody was 3.4 μM. The injection volume was 3 μL per analysis.

RESULTS AND DISCUSSION

The most commonly used approach for MS quantitation of proteins is surrogate peptide analysis in which a protein analyte is digested into peptides and a peptide is selected and quantified by MS. The measured quantity of the surrogate peptide reflects the amount of its precursor protein analyte. Our CMS method for protein absolute quantitation also adopts the protein digestion step but quantifies the surrogate peptide using MS combined with electrochemistry (EC). To test if CMS is applicable to quantify peptides from a protein digest, we first examined the feasibility of measuring peptides from a mixture. In our experiment, three tyrosine-containing peptides, GGYR, DRVY, and Arg^8-vasotocin (sequence: CYIQNCPRG-NH_2, 1−6 disulfide bond) were mixed together to serve as a mixture sample which underwent reversed-phase LC separation under gradient elution conditions, online electrochemical oxidation and subsequent MS detection. Extracted-ion chromatograms (EIC) of +2 ions of the three peptides after UPLC separation are shown in Figures 1a−c, respectively. The injection volume of mixture was 3 μL

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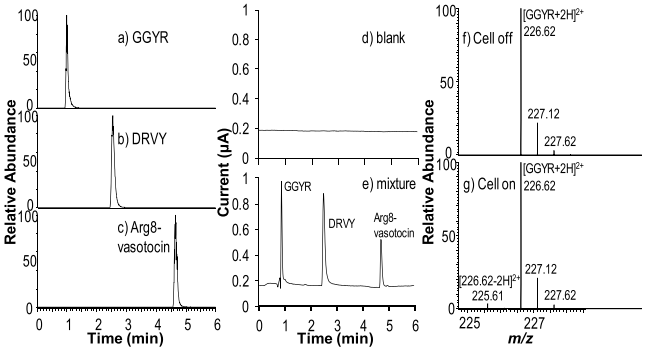

Figure 1. Extracted ion chromatograms of (a) GGYR, (b) DRVY, and (c) Arg^8-vasotocin. Electric oxidation current diagrams are shown due to the oxidation of (d) a solvent blank and (e) the peptide mixture. ESI-MS spectra of GGYR was recorded (f) when the cell was turned off and (g) when the cell was turned on (applied potential: + 0.95 V). The +2 ion of the oxidized GGYR product was observed at m/z 225.6 in g).

(injected amount: 60 pmol GGYR, 60 pmol DRVY, and 75 pmol Arg^8-vasotocin). At the same time, oxidation current peaks corresponding to different peptides were recorded (Figure 1e). In contrast, no oxidation current peaks were observed in Figure 1d from a blank solvent sample under the same oxidation potential of +0.95 V, indicating that those peaks observed in Figure 1e are results of electrochemical oxidation of the three peptides. Indeed, the online MS detection following the electrochemical oxidation confirmed that those peaks are from the peptide as labeled in Figure 1e. For instance, before electrolysis (Figure 1f, no potential was applied to the cell), the +2 ion of GGYR was observed at m/z 226.6. When +0.95 V was applied to the cell for oxidation (Figure 1g), a peak at m/z 225.61 arose, corresponding to +2 ion of the oxidized peptide product (one tyrosine residue loses two hydrogens and two electrons (z = 2) to form semiquinone upon oxidation; as a result, 2 Da mass shift occurs to the peptide ion[23]). The electrochemical oxidation consumed peptide and therefore its intensity dropped after electrolysis. Due to the dependence of ion intensity on concentration, the relative peptide ion intensity change would suggest the relative concentration change (i.e., the oxidation yield of the peptide). As shown in SI Figure S1, the integrated EIC peak area for the GGYR ion (m/z 226.6, the +2 ion) at +0.95 V was smaller than that at 0 V by 15.6%. This result indicates that the GGYR oxidation yield was 15.6% (SI Table S1). Meanwhile, by applying the Faraday's Law with the integrated current peak area, the amount of GGYR that was oxidized was shown to be 9.9 pmol. Therefore, the total amount of GGYR was measured to 63.5 pmol by CMS. A triplicate measurement gave the average amount of GGYR to be 63.8 ± 0.6 pmol, which turned out to be close to the injection amount (60 pmol) with the measurement error of 6.3%. Following the same procedure, the quantitation errors for the other two peptides, DRVY and Arg^8-vasotocin, were 2.7% and 1.9% (Table S1, Supporting Information), respectively.

With the success in using CMS to quantify peptides in mixture, we started absolute protein quantitation, using the surrogate peptide approach. β-casein, a commonly found phosphoprotein in mammalian milk (224 amino acids, sequence shown in SI Table S2), was tested by CMS. The protein was first tryptic digested and a tyrosine-containing peptide AVPYPQR was separated out by chromatography for electrochemical oxidation and quantified by our method. Without oxidation (Figure 2a), +2 ion of AVPYPQR was observed at m/z 415.7. As shown in Figure 2b, after electrolysis, a peak corresponding to +2 ion of the oxidized AVPYPQR product was observed at m/z 414.7. EICs of the +2 ion of AVPYPQR (m/z 415.7) from 3 μL of the 30 μM β-casein digest (90 pmol) without and with oxidation are shown in Figure 2c and d, respectively. The integrated peak area of m/z 415.7 shown in Figure 2d was smaller by 5.9% compared to the same peak in Figure 2c, suggesting that the oxidation yield for AVPYPQR was 5.9% (see detailed data in SI Table S3). Figure 2f shows the electric current peak from oxidation of AVPYPQR (as a control, no oxidation current peak was

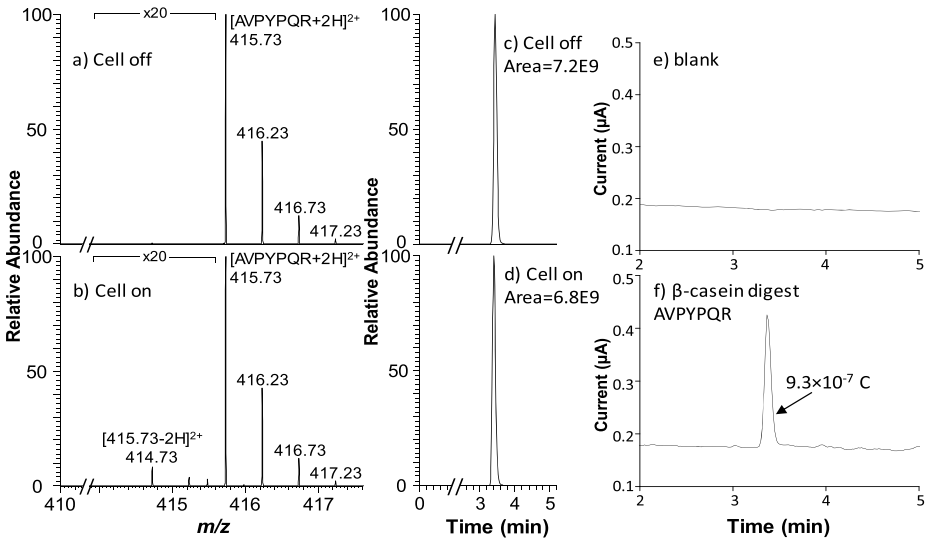

Figure 2. MS spectra of AVPYPQR from the β-casein tryptic digest (a) when the cell was off and (b) when the cell was turned on (applied potential: + 0.95 V). The oxidation product of AVPYPQR was detected at m/z 414.7. EICs of AVPYPQR were acquired (c) when the cell was off and (d) when the cell was turned on (applied potential: + 0.95 V). Electric current diagrams were collected from (e) blank solvent and (f) the oxidation of AVPYPQR.

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observed in Figure 2e from solvent blank under the same oxidation potential). The amount of oxidized AVPYPQR was calculated to be 4.8 pmol, based on the integration of the current peak area. Therefore, the measured amount of AVPYPQR was 80.1 pmol (SI Table S3). In a triplicate measurement, the averaged quantity of this peptide measured by CMS was 79.1 pmol (SI Table S3). For confirmation, an isotope-labeled peptide AVPYPQR^ (labeled at arginine, ^{13}C6, ^{15}N4) was purchased and used as an internal standard to quantify AVPYPQR in the same β-casein digest. By this traditional isotope dilution method, AVPYPQR in β-casein digest was measured to be 78.9 pmol (SI Figure S2). This value is in excellent agreement with the quantity of 79.1 pmol from our CMS method (only differed by 0.3%), confirming the possibility of CMS for quantitation. In comparison, traditional absolution quantitation based on the use of isotope labeled peptide standards would need 3−5 data points (i.e., 3−5 sample injections) to construct the calibration curve for quantitation. In comparison, our CMS method is faster, as it actually just needs two injections for quantifying one sample. Furthermore, the quantity of 79.1 pmol from our CMS measurement is also close to the initial amount of β-casein (90 pmol) that was used to generate the digest. The measurement error of −12.1%, defined as the difference between the moles of AVPYPQR peptide and the moles of β-casein (determined by weight), indicates that there is likely a slight sample loss during the process for tryptic digestion of proteins to peptides.[28]

Another protein, apomyoglobin (153 amino acids, sequence is shown SI Table S2), was also analyzed by CMS. After proteolytic digestion, YKELGFQG, a Tyr-containing and electro-oxidizable peptide, was identified and separated by LC/MS analysis, which can be used for quantitation with our method. Without oxidation (SI Figure S3a), +2 ion of YKELGFQG was observed at m/z 471.2. As shown in SI Figure S3b, after electrolysis, a peak corresponding to +2 ion of the oxidized YKELGFQG product was observed at m/z 470.2, due to two hydrogen losses from tyrosine oxidation. EICs the +2 ion of YKELGFQG (m/z 471.2) from 3 μL of 25 μM apomyoglobin digest (75 pmol) without and with oxidation are shown in SI Figures S3c and S 3d, respectively. The peak area of m/z 471.2 decreased by 0.98% upon oxidation (comparing SI Figure S3c and d), showing that the yield of peptide YKELGFQG oxidation was 0.98% (SI Table S4). In addition, the current peak as a result of oxidation of YKELGFQG was detected (SI Figure S3f). The amount of oxidized YKELGFQG was calculated to be 0.68 pmol, based on the current peak integration. Therefore, the measured amount of YKELGFQG was 68.9 pmol and a triplicate measurement gave an averaged quantity of 66.6 pmol (SI Table S4). Assuming that one molecule of protein apomyogloin produces one molecule of peptide YKELFQG (note that further cleavage between K and E residues in YKELFQG by trypsin was negligible, probably due to the influence of adjacent acidic residue E which forms a salt bridge with K residue[29]), the measured protein amount is therefore 66.6 pmol. This value is quite close to the amount of protein of 75.0 pmol (11.1% error, SI Table S4) for generating the 3 μL of the protein digest that was analyzed. This result further confirms the viability of using CMS for protein quantitation.

With the success in quantifying standard model proteins, we took a step further to use our CMS method for the quantitation of a biological sample of KaiB protein isolated from *E. coli* (sequence shown in SI Table S2). KaiB protein is a circadian clock protein in cyanobacteria. The circadian clock is an endogenous timekeeping mechanism that provides many advantages for life in a rhythmically changing environment.[30] The gene expression, metabolism, physiology, and behavior of almost all light-perceiving organisms living on earth are governed by a circadian (~24 h) clock, which anticipates the daily rhythm of the sunlight and the ambient temperature.[31,32] Disruption of the circadian clock in humans is correlated with many health issues, such as cancer, heart attacks, obesity, diabetes, fatigue, mood disorders, and most notably jet lag.[33−37] The cyanobacterial circadian clock is a simple model system of the human circadian clock. The cyanobacterial circadian clock regulates more than 30% of its gene expression which affects the physiology of the cell.[35] In cyanobacteria, the amounts of the gene expression products oscillate with a 24 h period. Till now, there is no commercially available protein standard for the KaiB protein quantitation. The quantitation of the transcription products (RNA) has been reported[38,39] but the absolute quantitation of the translation products of protein KaiB has not been reported yet. Because the proteins are the major determinant of the cell physiology, the timely measurement of the protein amount is significantly important to understand the circadian regulation of the gene expression and its physiology of cyanobacteria.

In our experiment, the cloning and purification of KaiB were performed as described previously with minor modifications[24,25] and details are shown in the Experimental Section. After digestion of KaiB protein isolated from *E. coli*, a Tyr-containing peptide VLIGLDLLYGELQDSDDF could be separated out and quantified by CMS. Without oxidation (Figure 3a), the +2 ion of VLIGLDLLYGELQDSDDF was observed at m/z 1013.0. When the electrochemical cell was turned on for oxidation (Figure 3b), +2 ion of the oxidized peptide product arose at m/z 1012.0. The integrated area for the m/z 1013.0 peak after electrochemical oxidation was smaller by 6.5% (note that there is a little contribution to the

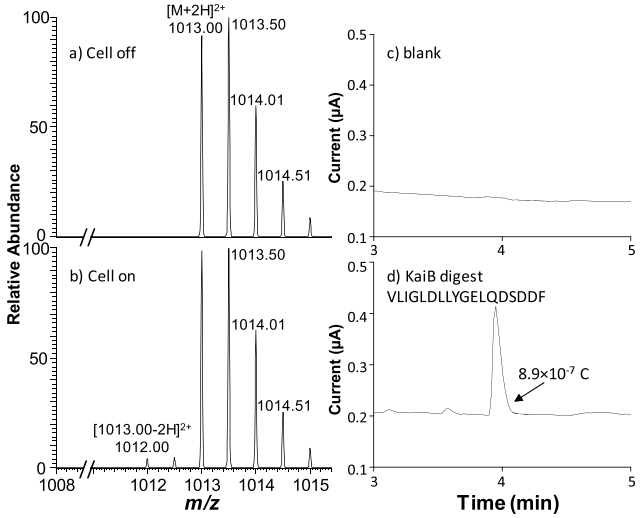

Figure 3. MS spectra of VLIGLDLLYGELQDSDDF from the KaiB tryptic digest (a) when the cell was off and (b) when the cell was turned on (applied potential: + 0.95 V). The oxidized product peak was detected at m/z 1012.0 (+2 ion) in (b). Electric current response curves are shown, due to oxidation of (c) solvent blank and (d) VLIGLDLLYGELQDSDDF.

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peak of m/z 1013.0 from the third isotopic peak of m/z 1012.0, which was corrected for the oxidation yield calculation; see details in SI Table S5), compared to the same peak before oxidation, which indicated that the oxidation yield was 6.5%. Meanwhile, the electric current as a result of oxidation of VLIGLDLLYGELQDSDDF was observed (shown in Figure 3d). The amount of VLIGLDLLYGELQDSDDF that underwent oxidation was calculated to be 4.6 pmol, based on the integration of the current peak area. Therefore, the amount of peptide measured by CMS was 71.3 pmol (the averaged value from a triplicate measurement: 73.3 pmol, SI Table S5). For comparison, an isotope-labeled peptide VLIGLDLLYGELQDSDDF (labeled at leucine, $^{13}C6$, ^{15}N) was purchased and used as an internal standard to quantify the same KaiB protein digest. The measured quantity for VLIGLDLLYGELQDSDDF by this isotope dilution method was 71.1 pmol (see data SI Figure S4). Again, the peptide quantity measured by two different methods are in excellent agreement (differed by 3.0%), providing validation of this CMS quantitative analysis approach. Based on our Bradford assay of the initial KaiB protein sample, the total amount that was used to produce the KaiB digest was estimated to be 84 pmol (28 μM, 3 μL). The moles of the surrogate peptide VLIGLDLLYGELQDSDDF measured by our CMS is close to the moles of KaiB protein, with the measurement error of −12.8% (likely due to sample loss during the digestion/desalting processes).

Besides that, an alternative comparison by quantifying two tryptic digested peptide fragments from the same KaiB digestion sample was conducted to further validate this method. Two peptide fragments NILEVEFQGVYALK and VLIGLDLLYGELQDSDDF were selected and quantified from the same injection run in a separate trial (SI Table S6). While VLIGLDLLYGELQDSDDF was determined by CMS to be 17.6 pmol, another peptide NILEVEFQGVYALK was measured to be 18.5 pmol, and their quantities were in good agreement (4.9% difference). Such a redundancy experiment could help to confirm the robustness and reproducibility of the CMS method.

To further test the capability of the CMS method, Vectibix (panitumumab, approximate molecular weight 147 kDa, sequence shown in SI Table S2), a recombinant human IgG2 kappa monoclonal antibody binding specifically to the human epidermal growth factor receptor (EGFR) was also tested and quantified in this experiment. After antibody was acetone precipitated and on-pellet digested, LLIYDASNLETGVPSR, a Tyr-containing peptide from light chain was identified and separated by LC/MS analysis, which can be used for quantitation by our method. Without oxidation (SI Figure S5a), peptide LLIYDASNLETGVPSR was observed at m/z 874.5 (+2 ion) and chosen as the surrogate peptide. As shown in SI Figure S5b, after electrolysis, the oxidized product of LLIYDASNLETGVPSR (+2 ion) was detected at m/z 873.5, due to two hydrogen losses from tyrosine oxidation. SI Figure S5c and d show the EIC the +2 ion of LLIYDASNLETGVPSR (m/z 874.5) from 3 μL of 3.4 μM IgG2 antibody digest (10.2 pmol) without and with oxidation, respectively. Note that one IgG2 molecule contains two identical light chains in which the surrogate peptide fragment is located. Therefore the theoretical amount of surrogate peptide was 20.4 pmol per injection by calculation. The oxidation yield for LLIYDASNLETGVPSR was suggested to be 8.2% (SI Table S7), by comparing the peak area of m/z 874.5 before and after oxidation (SI Figure S5c and d). Meanwhile, the amount of the oxidized LLIYDASNLETGVPSR was calculated to be 1.28 pmol, based on the area integration of electric current peak observed from peptide oxidation (SI Figure S5f). Therefore, the measured amount of LLIYDASNLETGVPSR was 15.7 pmol (triplicate average: 15.0 pmol). Compared to the theoretical amount (20.4 pmol) of this surrogate peptide, the measurement error was −26.4%. The error is relatively bigger than those shown above for other proteins, probably due to the large size of this antibody which may lead to difficulty in its digestion. Indeed, sample loss up to 32% during antibody digestion was reported before.[40] Other possible contributing factors affecting the digestion efficiency are the low antibody concentration, multiple preparation and digestion steps, artificial degradations, and rich disulfide bonds structure, etc..[28,40−43] A possible solution to alleviate the sample losses could be the use of combined enzymes such as Lys-C and trypsin to increase digestion efficiency. Nevertheless, the CMS method has demonstrated its capability to absolute quantitation of large biomolecules and potential practical utility in drug development. Although quantitation of mainly pure protein samples were demonstrated in this study, our CMS method is expected to be applicable for quantifying proteins in mixture as it allows selective electrochemical oxidation of tyrosine-containing surrogate peptides.[23] Such an experiment is under way.

CONCLUSIONS

In this study, we demonstrated the proof-of-concept of using coulometric mass spectrometry approach to quantify proteolytic surrogate peptides with the aim of quantifying proteins. Several proteins were successfully quantified by using this CMS method, after digestion. The results measured by CMS were very comparable to the results by isotope-labeling method. The advantage of this method is that neither standard peptide nor isotope-labeled peptide is in need for absolute protein quantitation. Our CMS experiment is virtually a typical bottom-up LC/MS proteomics experiment. With only addition of an EC component, quantitative information of proteins can be obtained, showing the power of our CMS method. It is also fast as no standard curve needs to be obtained for quantitation. Redundancy experiment for simultaneous quantifying two peptides from one protein digest is possible. Future work will be focused on applying CMS method for real-world applications in absolute protein quantitation of biological samples.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c01151.

Additional MS spectra, experimental setup, and data (PDF)

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Notes

The authors declare no competing financial interest.

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