ISOBARIC LABELING PROTEOMICS FOR HIGH-THROUGHPUT INVESTIGATION OF PROTEIN CORONA ORIENTATION

Nara Liessi[1], Luca Maragliano[2,3],Valentina Castagnola[2,4], Mattia Bramini[2,5], Fabio Benfenati[2,4] and Andrea Armirotti[1]

Affiliations:

1) Analytical Chemistry Lab, Istituto Italiano di Tecnologia, Via Morego 30, 16163, Genova, Italy

2) Center for Synaptic Neuroscience and Technology, Istituto Italiano di Tecnologia, Largo Rosanna Benzi 10, 16132 Genova, Italy

3) Department of Life and Environmental Sciences, Marche Polytechnic University, Via Brecce Bianche, 60131, Ancona, Italy

4) IRCCS Ospedale Policlinico San Martino, Largo Rosanna Benzi 10, 16132 Genova, Italy

5) Department of Applied Physics, Faculty of Sciences, University of Granada, Spain

MATERIALS AND METHODS

Chemicals, materials and solvents

TMT sixplex Isobaric Mass Tagging Kit and Reagents were purchased from Thermo Scientific (Rockford, IL, USA). RapiGest™ reagent for enhancing enzymatic digestions of proteins was purchased from Waters (Milford, USA). All other chemicals and reagents used for sample preparation and LC-MS/MS analysis were purchased from Aldrich (Milano, Italy). Human plasma was purchased from Tebu-Bio (Milano, Italy). Few layers graphene (FLG) was prepared from pure graphite according to Gonzáles-Domínguez et al.[1]. Graphene Oxide (GO) was purchased from Sigma Aldrich (Catalog N°796034).

Material characterization

GO and FLG were characterized by Raman and transmission electron microscopy (TEM) imaging. Raman spectroscopy was performed using a 532-nm excitation laser (10% of maximum power) and a 20X objective lens to collect Raman emission. The spectra were recorded on a dried droplet, averaged over 5 measurements. TEM imaging was performed on a JEOL JEM1011 (JEOL USA, Inc, Peabody, MA) operating at 100 kV.

Protein corona formation, labeling with Tandem Mass Tags (TMT) and digestion with trypsin

Commercial human plasma was centrifugated at 20000 x g, at 4 °C for 20 min. Graphene Oxide (GO), and Pristine Graphene (FLG) were suspended in PBS (phosphate-buffered saline, pH=7,4) at a final concentration of 10 mg/mL. GO and FLG were then incubated with human plasma at a final 1 mg/mL concentration for 60 h at 37 °C, under shaking. At the end of incubation, the samples were centrifuged at 20000 x g, RT for 20 min, the supernatant was removed and the pellet washed three times with PBS. The

resulting pellets was resuspended in 50 µL of 100 mM triethyilammonium bicarbonate (TEAB), pH 8 and 13 µL of TMT-126 reagent (reconstituted immediately before use with acetonitrile) were added. Labeling reaction occurred then 1h at +4 °C under shaking. After centrifugation at 20000 x g at room temperature (RT) for 20 min, the supernatant was discarded and the pellet washed twice with TEAB. The protein corona was then denatured and detached from the material by adding 50 µL of a denaturing buffer consisting of urea 8 M, 1% sodium dodecylsulfate (SDS) and 10 mM dithiothreitol (dDTT) dissolved in 100 mM TEAB, pH 8. The samples were then incubated at 55 °C for 1 h, under shaking. After centrifugation at 20000 x g for 20 min at RT, the supernatant was collected and TMT-127 reagent (13 µL, reconstituted immediately before use with acetonitrile) was added and incubated for 1 h, RT under shaking. The protein cysteines were then alkylated with iodoacetamide (20 mM final concentration, 20 minutes at RT in the dark). At the end of the reaction, 1.2 mL of pre-chilled (-20 °C) acetone were added to the samples, that were then kept O/N at -20 °C. After centrifugation at 20000 x g, 4 °C, 20 min, the supernatant was removed and protein pellets were washed with 200 µL of methanol. The protein content was then solubilized and 0.2% (w/v) Rapigest [2] in 50 mM ammonium bicarbonate, pH 8. Trypsin was then added (1 µg) and the samples were digested ON at 37 °C, under shaking. Prior to analysis, Rapigest was hydrolyzed with trifluoroacetic acid (0.5% for 45 min at 56 °C). After digestion, the surnatant was collected end evaporated by vacuum centrifugation. At the time of LC-MS/MS analysis, the peptides were dissolved in 30 µL of 3% acetonitrile in water (+0,1 % formic acid).

Data acquisition

Tryptic peptides were analyzed by high-resolution LC-MS using a UPLC NanoAcquity chromatographic system (Waters, Milford, MA, USA) coupled with a TripleTof 5600+ mass spectrometer (Sciex, Warrington, UK) equipped with a NanoSpray III ion source. The peptides were loaded and desalted on a trapping column 0.15 X 50 mm (Waters) for 3 minutes then moved on a Eksigent 0.3 X 200 mm C18 micro column. Eluents were A (water + 0.1 % formic acid) and B (acetonitrile +0.1% formic acid). Injection volume was set to 5 µL (Full Loop), flow rate was set to 5 µL/min, the column was kept at 35 ºC, samples were eluted with the following gradient: 0,0 -1,0 min 2 % B; 1,0 - 60,0 min 2 to 30 % B; 60,0 – 65,0 min 30 to 90 % B; 65,0 – 70,0 min 90% B and 70,0 – 71,0 min 90 back to 2 % B. The column was then reconditioned for 9 min. The total run time was 80 min. The spectra were acquired in ESI+, data-dependent acquisition (DDA) mode. The scan range was set from 350 to 1250 m/z for MS and from 100 to 1,500 m/z for MS/MS. Precursor ions with charge states 2 and 5 with intensity greater than 150 counts were selected for MS/MS. Collision energy profiles were set according to SCIEX recommended settings.

Data analysis

Raw data were converted into .mgf file format analyzed using Protein Pilot (SCIEX). MASCOT [3, 4] (MatrixScience Ltd, London, UK) was then used to search the mgf spectra files against the SWISSPROT *Homo Sapiens* database for proteomics. The following parameters were used for the search. Enzyme: Arg-C (corresponding to trypsin with missed cleavage at K). Fixed modifications: carbamidomethyl at C, TMT-6plex at K. Variable modification: methionine oxidation, phosphorylation at T and S. Precursor and fragment ion tolerance: 50 ppm. Venn diagrams (overlap analysis) were prepared with FUNRICH[5]. Multivariate data analysis was performed using the publicly available MetaboAnalyst tool [6]. The dataset of 46 common peptides (overlap of the three groups) was normalized by the median In/Out ratio of each peptide, Log transformed and Pareto scaled (mean-centered and divided by the square root of the standard deviation of each variable). Heatmap analysis was performed using the euclidean distance measure with Ward clustering algorithm. Gene enrichment analysis was performed using FUNRICH open access tool [7].

Docking calculations

A sheet of GO was generated starting from a set of structures of the CSIRO database [8], each with different, random coverages of epoxy and hydroxyl groups. An oxygen concentration of about 9.5% was obtained by randomly removing some of the groups. Docking calculations were performed using Patchdock and Firedock programs [9]. The following protein structures from the Protein Data Bank (PDB) were docked on the GO sheet: CBPN, PDB code 2nsm [10]; TRFE, PDB code 2hau [11]; HBA, PDB code 1hho [12]; HV323, PDB code 1ohq [13]; RL40, PDB code 1ubq [14]. **Figure 4** reports the docked pose that, for each protein, ranked as first.

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