MATERIALS

REAGENTS

- Dulbecco's phosphate-buffered saline 1X (DPBS, Invitrogen™).
- D-Glucose-$^{13}C_6$ (lyophilized powder, 99 atom % ^{13}C, Sigma-Aldrich®).
- 1 M triethylammonium hydrogen carbonate buffer, pH 8.5 (TEAB, Fluka)
- Bovine serum albumin (BSA, lyophilized powder, ≥ 96%, Fluka)
- Protein assay dye reagent concentrate (liquid, Bio-Rad) ▲ **CRITICAL** It is essential to verify the compatibility of Bio-Rad Protein assay with reagents used for proteins solution.
- 0.5 M tris-(2-carboxyethyl) phosphine hydrochloride, pH 7.0 (TCEP, Sigma-Aldrich®) **! CAUTION** TCEP should be handled with protective eyewear and gloves.
- Iodoacetamide (IAA, crystalline, ≥99%, Sigma-Aldrich®) **! CAUTION** IAA should be handled in a hood using gloves.
- Endoproteinase Glu-C from Staphylococcus aureus V8 (lyophilized powder, 500 U, Sigma-Aldrich®) **! CAUTION** Glu-C should be handled in a hood using protective eyewear and gloves.
- Ammonium acetate (NH_4Ac, solid, 98.0%, Fluka) **! CAUTION** NH_4Ac should be handled in a hood using protective eyewear and gloves.
- Magnesium chloride ($MgCl_2$, solid, ≥99.0%, Fluka)
- Sodium hydroxide (NaOH, pellets, 99%, AnalR®)
- Acetic acid (99.5%, Fluka) **! CAUTION** Acetic acid should be handled in a hood using protective eyewear and gloves.
- HPLC-grade water (CHROMASOLV®, Sigma- Aldrich®)
- HPLC-grade acetonitrile (ACN, CHROMASOLV®, ≥99.9%, Sigma-Aldrich®) **! CAUTION** ACN is harmful and highly flammable and should be handled in a hood using protective eyewear and gloves.
- Formic acid (98%, Fluka) **! CAUTION** Formic acid is corrosive and flammable and should be handled in a hood using protective eyewear and gloves.
- EDTA-free Protease inhibitor cocktail tablets that contain: Aprotinin, Bestatin, Calpain inhibitor I, Calpain inhibitor II, Chymostatin, E 64,

Leupeptin, α-2 Macroglobulin, Pefobloc SC, Pepstatin, PMSF, TLCK-HCl, Trypsin inhibitor (chicken, egg white), Trypsin inhibitor (soybean) (Roche).

EQUIPMENT

- Amicon Ultra-0.5 ml, Ultracel®3K membrane (Millipore™)
- Spectrophotometer (GE Healthcare)
- SpeedVac concentrator (Savant)
- Shaker
- Waters 600E HPLC system (Millipore Corporation, USA) equipped with a TSK gel boronate-5PW matrix chromatographic column (7.5 cm length x 7.5 mm inner diameter, i.d., 10 µm particle size, Tosoh Biosciences)
- MacroSpin Column™ C_{18} (Harvard Apparatus, Holliston, MA, USA)
- Waters NanoAquity HPLC system (Milford, MA) equipped with a helium degasser
- A home-made precolumn of 100 µm i.d. x 18 mm length, packed with 200 Å (5 µm) Magic C_{18} particles (Michrom)
- A home-made analytical column gravity-pulled of 75 µm i.d. x 150 mm length, packed with 100Å (5 µm) Magic C_{18} particles (Michrom)
- Hybrid linear ion trap-Orbitrap mass spectrometer (Thermo Fischer, San Jose, CA)

REAGENT SETUP

Biological sample collection Acquire appropriate ethical approval from the local research ethics committee (or appropriate group) before collection of any biological sample. **! CAUTION** Apply all relevant ethical guidelines during collection of all samples. Samples should be collected by a clinician or study nurse. It is good practise to screen the devices used for obtaining the samples together with the containers used for sample collection and storage. **! CAUTION** Some biofluids can provide a potential infection risk; perform all work with appropriate personal protection equipment including gloves and glasses. Collection, processing and storage of biological samples from volunteers and patients are performed under GLP.

Glucose labelling [$^{13}C_6$] D-Glucose is dissolved in DPBS at 25 mg/ml (134.33 mM). ▲ **CRITICAL** The solution is stored at 2-8 °C.

Bradford assay BSA powder, used as a calibration standard, is dissolved in DPBS to reach a concentration of 5 mg/ml. ▲ **CRITICAL** The solution is stored at -80 °C.

Glu-C enzymatic digestion ! CAUTION Reagents are irritants, wear appropriate personal protective equipment and perform the experiment in a hood. TEAB is diluted in water to reach the concentration of 0.5 M. ▲ **CRITICAL** TEAB can be stored at 4°C for a period no longer than one month. TCEP is diluted in water at 0.05 M (2 µL of TCEP in 20 µL of water). Iodoacetamide is dissolved in water at 0.4 M. ▲ **CRITICAL** TCEP and IAA must be prepared daily. IAA solution should be kept in the dark. Endoproteinase Glu-C is reconstituted in 0.5 M pH 8.5 TEAB at 1 mg/ml.▲ **CRITICAL** Glu-C is stored in aliquots at -80°C. **! CAUTION** Glu-C is an irritant, wear appropriate personal protective equipment and perform the experiment in a hood.

Mobile phase solutions for BAC analysis Fractionation of peptides is carried out by boronate affinity chromatography using:**-**

Mobile phase A: 200 mM NH_4Ac, 50 mM pH 8.1 $MgCl_2$ (adjusted with 0.1 M NaOH) in water.

Mobile phase B: 100 mM acetic acid in water pH (~ 3).

▲ **CRITICAL** Both solutions are stored at room temperature and must be prepared fresh every week. In addition, both solutions must be set at the specified pH values before any BAC analysis. **! CAUTION** NH_4Ac and Acetic acid are irritants, wear appropriate personal protective equipment and perform the experiment in a hood.

Desalting treatment and LC–MS/MS analysis Both eluted fractions from BAC are evaporated and reconstituted in a Washing solution prior to the following desalting/concentration step with C_{18} macro spin column.

Washing solution: 5% ACN/0.1% formic acid in water.

Eluting solution: 50% ACN/0.1% formic acid in water.

RP–LC solvent A: 0.1% formic acid in water.

RP–LC solvent B: 100% ACN/0.1% formic acid in water.

▲ **CRITICAL** All solutions are stored at room temperature and should be prepared fresh every month. **! CAUTION** Chemicals are highly flammables and must be handled in a hood.

EQUIPMENT SETUP

BAC The column is equilibrated by running mobile phase A for 40 min approximately to reach its optimum efficiency and resolution.

Sample injection volume is set to 50 µL (full loop) per run and peptides are eluted by an isocratic chromatographic method as shown in **Table 1**.

▲ **CRITICAL** All steps are performed at room temperature. Column is washed by running mobile phase A for 30 min or injecting 0.1 M NaOH at the end of total analyses.

LC system and Mass Spectrometer Nanoflow LC is carried out on a system that consists in a home-made pre-column (100 µm inner diameter and 18 mm in length) packed with C_{18} resin, where peptides are initially loaded at 3 µl/min in water/ACN (95/5 vol/vol) with 0.1% formic acid (*v/v*), and of a home-made gravity-pulled (75 µm inner diameter and 15 cm in length) analytical column packed with C_{18} resin directly interfaced to the mass spectrometer operated in positive ion mode. The electrospray ionization voltage of 1.6 kV is applied through a liquid junction using a platinum wire connected to a micro-tee union between the pre-column and the analytical column. Once retained, peptides are eluted using an ACN gradient at a flow rate of 220 nl/min with RP-LC solvent A, water and 0.1% formic acid; and RP-LC solvent B ACN and 0.1% formic acid. The followed HPLC gradient is described in **Table 2**.

The mass spectrometer used in this study is a hybrid linear ion trap-Orbitrap (Thermo Fisher, San Jose, CA) that, due to its high-resolution power, mass accuracy and scan speed, leads to an increase in sequence coverage and identification as well to more quantifiable peptides. The MS analysis for both HCD–MS2 and CID–MS3 in neutral loss scanning are separately carried out setting the parameters showed in **Table 3**. After the acquisition of a full-scan survey mass spectrum, three full-scan MS/MS spectra are acquired sequentially on the three most abundant ions detected in the MS1, for HCD, while five most abundant ions are selected on the MS1 scan to be fragmented for the MS3 analysis.

PROCEDURE

Prepare and incubate sample with [$^{13}C_6$]-Glucose ● TIMING 1 day

1I Measure protein amount using Bradford assay and a standard calibration curve with BSA.

2I Resuspend a volume or amount of sample representing 1 mg of proteins in 500 µL DPBS and incubate with 30 mM [$^{13}C_6$]-glucose, at 37°C for 24 h.

3I After incubation, remove glucose and salts by ultra-filtration using Amicon Ultra-0.5 devices, with a 3KDa nominal molecular weight cut-off, and recover proteins in 0.5 M, pH 8.5, TEAB.

4I Measure protein amount using Bradford assay and a standard calibration curve with BSA.

■ **PAUSE POINT** The glycated proteins are stable for several months when stored at -20 °C.

Endoproteinase Glu-C digestion of proteins ● TIMING 8h

5I Take a protein amount of 1 mg for the enzymatic hydrolysis with Glu-C.

6I Reduce disulfide bonds by protein incubation 50 mM TCEP in water (20 µL) for 1 h at 60°C. ▲ **CRITICAL STEP** Prepare TCEP solution just before incubation.

7I Alkylate sulfhydryl groups of protein cysteine residues by incubating sample with 400 mM IAA in water (10 µL) and shaking for 30 min at room temperature and dark conditions. ▲ **CRITICAL STEP** Prepare alkylating buffer just before use and keep it in the dark.

8I After blocking of cysteine residues with IAA, add 100 µL of 1 mg/ml solution of endoproteinase Glu-C to the proteins solution (ratio of 1:10 *w/w*) and incubate overnight at 37°C. ▲ **CRITICAL STEP** Prepare endoproteinase Glu-C solution just before use. The selection of this enzyme is supported by the null influence of glucose attachment sites, reducing the proportion of missed cleaved peptides.

9I Evaporate the peptides solution using a concentrator/speed vacuum and reconstitute the dried sample in 50 µL mobile phase A for fractionation of glycated peptides.

■ **PAUSE POINT** The peptides solution is stable for several months when stored at -20 °C.

Enrichment of glycated peptides by boronate affinity chromatography ● TIMING 2h

10| Select the isocratic chromatographic method shown in the EQUIPMENT SETUP section (**Table 1**) for the affinity separation of non-glycated and glycated fractions. Briefly, from 0 to 10 min, glycated peptides are retained on the stationary phase by esterification between the 1,2 *cis*-diol groups of glucose moieties and the hydroxyl groups of boronate ligands under alkaline conditions, with the consequent elution of non-glycated species; from 10 to 20 min, glycated peptides are eluted by decreasing the pH with 100 mM acetic acid solvent; from 20 to 30 min, the column is equilibrated to the initial conditions. ▲ **CRITICAL STEP** It is important to equilibrate the column prior to the sample injection. For this purpose, pass mobile phase A through the column for 10 min.

11| Inject 50 µL of reconstituted peptides in the Waters 600E HPLC system equipped with the boronate affinity column at room temperature. ▲ **CRITICAL STEP** Clean potential contaminants attached to the column by injecting a control sample or 0.1 M NaOH solution.

12| Recover the non-glycated and glycated fractions separately; then, evaporate both solutions with a concentrator/speed vacuum and reconstitute the dried samples in 400 µL of RP-HPLC solvent A.

■ **PAUSE POINT** Both fractions are stables for several months when stored at -20 °C.

Peptide desalting and concentration ● TIMING 1h

13| Desalt and pre-concentrate the reconstituted peptides using C_{18} Macrospin columns prior to LC-MS/MS analysis. These devices are preconditioned with 400 µL eluting solution and activated with 400 µL of washing solution. Pass samples through the column, wash with 400 µL of washing solution and then elute and recover with 400 µL of eluting solution. ▲ **CRITICAL STEP** Washing step is very important in

order to remove salts and other contaminants presents in the sample. Make it at least 3 times.

14I Evaporate the peptides using a concentrator/speed vacuum and reconstitute with the correct volume of RP-HPLC solvent A according to an estimated concentration of 3 µg of peptides/µL for the following MS analysis.

■ **PAUSE POINT** Desalted and concentrated samples are stables for several months when stored at -20 °C.

LC-MS/MS detection of glycated peptides ● TIMING 3h per sample

15I Analyze the samples with the hybrid linear ion trap-Orbitrap mass spectrometer by electrospray ionization in positive ion mode, coupled with a Waters NanoAquity HPLC system equipped with a helium degasser applying the following instrument parameters. Peptide mixtures are trapped on a home-made 100-µm inner diameter x 18-mm long pre-column packed with 200 Å (5 µm) Magic C_{18} particles. Peptide fractionation is carried out on a home-made gravity-pulled 75- µm inner diameter x 150-mm long analytical column packed with 100 Å (5 µm) C_{18} particles, and directly interfaced to the mass spectrometer. An estimated amount of 0.1 µg of glycated peptides is loaded on the pre-column at the flow rate of 3 ml/min in 5% ACN/ 0.1% FA. Chromatographic separations are done as described in the EQUIPMENT SETUP section (**Table 2**).

16I Analyze the samples in the mass spectrometer using two orthogonal data-dependent methods over the entire chromatographic profile: MS2 with high-energy collisional dissociation as activation mode in the octopole at the rear of the C-trap, and MS3 with CID as fragmentation mode in the linear trap, for the neutral loss scanning. MS analysis is automatically performed during the chromatographic separations. The setting parameters selected for each MS operating mode are indicated below.

(A) **HCD–MS2 analysis**

Each Orbitrap full MS1 survey scan (with high accuracy) is followed by the activation of the three most abundant precursor ions detected in the full MS1

scan. The resolution for full MS scan is set to 60000 in a mass range of 400-1600 m/z, ACG 5 x 10^5, maximum ion time 100 ms; HCD-MS2 spectra are acquired with a resolution of 7500 with a precursor isolation window of 4 m/z units, an ACG of 2 x 10^5 at a maximum ion accumulation time of 500 ms. Precursor ion with a charge state of +2 and higher are included for the data-dependent selection. Normalized collision energy is set to 50% and activation time to 30 ms.

(B) **CID–MS3 analysis in neutral loss scanning**

Each Orbitrap full MS1 survey scan (with high accuracy) is followed by an activation, on the linear trap, to promote the cleavage of the entire glucose moiety (-162.05 Da, which correspond to -81.02 and -54.01 m/z units for doubly and triply charged peptides, respectively) or an intermolecular rearrangement of the glucose unit (-84.04 Da, which correspond to -42.02 and -28.01 m/z units for doubly and triply charged peptides, respectively); isolation of the ion species in which one of the neutral losses is detected; and fragmentation of the isolated ion followed by ion trap detection. The MS3 neutral loss- setting consists of: full MS (AGC 3 x 10^4; maximum ion time 50 ms); MS3 (ACG 1 x 10^4; maximum ion time 100 ms; minimal signal required 500; isolation width 2 m/z units). Normalized energy collision is set to 35% with an activation time of 30 ms.

■ **PAUSE POINT** Archive raw analytical data for future use.

Protein/Peptide identification ● TIMING 2h

17| After spectral data acquisition, convert and process RAW files to mzXML and IDJ/MGF files using EasyprotConv with specific setting parameters for each MS operation mode.

18| Search the resulting data files against the UniProtKB/Swiss-Prot database using the Easyprot tool. The set of search parameters selected for each MS data is described in the INTRODUCTION section (see Data analysis subheading).

■ **PAUSE POINT** Archive processed data for future use.

Glycation quantification ● TIMING 1h

19| Quantify glycated peptides by calculation of ratios between signals corresponding to peptide ions containing the glucose modification (addition

of 162 or 168 mass units), which produce doublet signals in the MS-only survey spectrum. The analysis is based on the utilisation of SuperHirn software, which performs the assessment of glycation quantification in an automated manner. Further information about the analysis is presented in the INTRODUCTION section (see Data analysis subheading).

■ **PAUSE POINT** Archive processed data for future use.

- **TIMING**

  Steps 1-4, Prepare sample and incubate proteins with $^{13}C_6$-Glucose: 1 day.

  Steps 5-9, Endoproteinase Glu-C digestion of proteins: 8 h.

  Steps 10-12, Enrichment of glycated peptides by boronate affinity chromatography: 2h.

  Steps 13 and 14, Peptide desalting and concentration: 1 h.

  Steps 15 and 16, LC-MS/MS detection of glycated peptides: 3 h per sample.

  Steps 17 and 18, Protein/Peptide Identification: 2h.

  Steps 19 and 20, Glycation Quantification: 1h.

TABLES

TABLE 1 | Waters 600E HPLC gradient elution program applied for BAC analysis.

Time interval (min)	Gradient (%A - %B, *v*/*v*)
0' - 10'	100 – 0
10' - 20'	0 – 100
20' - 30'	100 – 0

TABLE 2 | Waters NanoAquity HPLC gradient elution program applied for LC-MS analysis.

Time interval (min)	Gradient (%A - % B, *v*/*v*)
0' - 55'	95 – 5
55' - 60'	65 – 35
60' - 65'	15 – 85

65' - 75'	85 – 15
75' - 90'	95 – 5

TABLE 3 | Hybrid linear ion trap-Orbitrap mass spectrometer program applied for MS analysis: MS2-HCD and MS3-CID neutral loss scanning.

Program parameters	Value
HCD–MS2	
Activation type	HCD (50% normalized collision energy)
MS1 resolution	60000
Full AGC target	5×10^5
MS1 scan range	400 - 1600 m/z
Max. ion time (MS1)	100 ms
MS2 resolution	7500
MS^n ACG target	2×10^5
Isolation window	4 m/z
Max. ion time (MS2)	500 ms
Activation time	30 ms
CID–MS3 Neutral loss	
Activation type	CID (35% normalized collision energy)
MS1 resolution	60000
Full AGC target	3×10^4
MS1 scan range	400 - 2000 m/z
Max. ion time (MS1)	50 ms
MS^n ACG target	1×10^4
Minimum signal threshold	500
Dynamic exclusion time	45 s
Isolation window	2 m/z
Max. ion time (MS2)	100 ms
Activation time	30