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Hydrodynamic size-based separation and characterization of protein aggregates from total cell lysates

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Abstract

Herein we describe a protocol that uses hollow-fiber flow field-flow fractionation (FFF) coupled with multiangle light scattering (MALS) for hydrodynamic size-based separation and characterization of complex protein aggregates. The fractionation method, which requires 1.5 h to run, was successfully modified from the analysis of protein aggregates, as found in simple protein mixtures, to complex aggregates, as found in total cell lysates. In contrast to other related methods (filter assay, analytical ultracentrifugation, gel electrophoresis and size-exclusion chromatography), hollow-fiber flow FFF coupled with MALS allows a flow-based fractionation of highly purified protein aggregates and simultaneous measurement of their molecular weight, r.m.s. radius and molecular conformation (e.g., round, rod-shaped, compact or relaxed). The polyethersulfone hollow fibers used, which have a 0.8-mm inner diameter, allow separation of as little as 20 µg of total cell lysates. In addition, the ability to run the samples in different denaturing and nondenaturing buffer allows defining true aggregates from artifacts, which can form during sample preparation. The protocol was set up using Paraquat-induced carbonylation, a model that induces protein aggregation in cultured cells. This technique will advance the biochemical, proteomic and biophysical characterization of molecular-weight aggregates associated with protein mutations, as found in many CNS degenerative diseases, or chronic oxidative stress, as found in aging, and chronic metabolic and inflammatory conditions.

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INTRODUCTION

Protein aggregation is a common biological phenomenon associated with several physiological and pathological conditions. Overall, protein aggregation indicates the cellular inability to maintain its own proteostasis, and it comprises the association of proteins into a larger assembly after the loss of their secondary, tertiary or quaternary structures, and it is often associated with the loss of their biological activity¹.

There is a keen interest in analyzing protein aggregates for several reasons:

- i. To elucidate how a protein's primary and secondary structure determines its propensity to associate into larger aggregates, under either physiological or pathological conditions such as in neurodegenerative diseases (Alzheimer's, Parkinson's and Huntington's diseases) associated with amino acid mutations^{2–5};
- ii. To map the post-translational oxidative modifications associated with pathologies that are known to induce protein aggregation, such as chronic inflammatory, metabolic and degenerative diseases and aging^{6–8}; and
- iii. To fractionate highly purified protein aggregates of different sizes and conformation in order to analyze the different cellular machinery associated with their refolding or disposal including chaperones, autophagy-related proteins and proteasome^{9–15}.

A recent review¹⁶ that discussed the current proteomics prefractionation methods highlighted the emerging role and advantages of FFF, a flow-based separation technique invented in the 1960s by Giddings^{17,18}. Flow FFF uses a 'flow' field to accomplish the separation, which consists of a stream of mobile phase that is applied orthogonally to the elution flow. Flow FFF offers high selectivity in terms of separating proteins with different diffusion coefficients (D); this coefficient is directly proportional to a protein's size, shape/conformation and molecular weight (MW), and thus it is unique to each protein¹⁶ (Fig. 1a,b). The separation occurs inside an empty capillary channel, and thus the shear stress exerted on the sample is minimal, allowing the sample components to preserve their biophysical features. This feature also allows for the separation to be performed in a sample-compatible/bio-friendly carrier solution. Flow FFF is also flexible in terms of channel design, a feature that led to the development of its miniaturized version called hollow-fiber flow FFF (abbreviated HF5). The reduced volume of the HF5 device involves the use of very low flow rates, and thus a reduced sample dilution, which also made possible the on-line HF5–mass spectrometry (MS) coupling^{19,20}.

The methodology described herein can be applied to the analysis (and relative quantification) of biological samples in which protein aggregation is very relevant to the pathogenesis of the disease. Amino acid mutation is one of the causes of protein aggregation in familiar forms of neurodegenerative diseases, including the aggregation of α -synuclein in Parkinson's disease^{2,5,21–23}, the aggregation of tau or β -amyloid in Alzheimer's disease^{3,24,25} and huntingtin protein in Huntington's disease^{26–30}. In addition, in chronic inflammatory and degenerative diseases and metabolic syndrome or diabetes, an increase in oxidative stress-related protein post-translational modifications, including glycation,

lipoxidation and carbonylation, is observed^{6,7,31–36}. These modifications are known to facilitate unfolding and protein aggregation^{6–8}. Last, any disease or condition that decreases the proteasome or the endosomal and lysosomal processing activity, the autophagic mechanisms or the chaperone folding machinery will increase protein aggregate and huntingtin protein in gation^{9,10,13,37–44}. In all these conditions, the ability to purify protein aggregates and to analyze their biophysical properties will enhance our knowledge of the pathogenesis of these diseases.

Development of the protocol

Recently, HF5 coupled online with UV and MALS detection was successfully used to conduct a stability study on a protein used for drug delivery: AvidinOX⁴⁵. The HF5 method that was developed for this purpose was successful in fractionating and calculating the absolute MW of the separated species, the AvidinOX monomer, dimer and tetramer, proving that the protein oligomers were stable under both native and denaturing conditions. Over the past 20 years, HF5 was successfully used to separate protein mixtures^{46,47}, including selected serum proteins under native conditions, and high- and low-density lipoproteins from human plasma samples^{19,48–50}, as well as to enrich low-abundance high-density lipoproteins in blood serum^{51,52}. HF5 was also used as a separation method for glycoproteins from bacterial lysates⁵³ and as a prefractionation method, in combination with complementary separation techniques such as capillary isoelectric focusing; it was also coupled on-line with MS for the proteomic analysis of bacteria (*Bacillus subtilis*, *Corynebacterium glutamicum* and *Escherichia coli*)⁵⁴ and human urinary proteins⁵⁵. The technique was also successfully used for the separation of prion particles and for the determination of the relationship between their size and their infectivity⁵⁶. However, so far FFF has not been used to separate complex proteins aggregates, as observed in total cell lysates.

Here we present a protocol based on HF5 coupled online with UV and MALS detection, which is used to separate high-MW (HMW) protein aggregates from whole-cell lysates and to determine their r.m.s. radius, absolute MW, conformation, shape and solubility.

The protocol is an improvement on an already published HF5-UV-MALS method⁴⁵, and it was adapted to separate complex protein mixtures, as found in total eukaryotic cell lysates. As the proteome sample reported here is highly more complex than the AvidinOX formulation or a mixture of lipoproteins or the bacterial proteome, some HF5 method parameters were adapted to allow a single-step extensive characterization of the cellular proteome samples.

Application of the protocol

To set up the methodology, we induce protein aggregation by treating the cells with Paraquat (PQ), which reproduces the type of protein aggregation observed in chronic inflammatory and degenerative conditions and aging^{6–8}.

Experimental design

The procedure is summarized in Figure 1c, and it consists of the following stages:

- i. *Sample preparation (Steps 1–10):* This stage includes cell culture, PQ treatment used to induce oxidative damage and subsequent protein aggregation, cell lysis and protein quantification.
- ii. *Assessing the efficacy of the PQ treatment:* This stage includes performing protein carbonyl spectrophotometric assays and SDS-PAGE on the total cell lysates. The amount of protein carbonyl should increase proportionally to the PQ concentration. After the PQ treatment, increased amounts of HMW protein aggregates are expected in the lanes of the SDS-PAGE (progressively darker color), as well as near (or inside) the stacking gel zone.
- iii. *Fractionation and characterization of the total cell lysates under native conditions:* The purpose of this stage is to isolate HMW protein aggregates. The experimental setup includes HF5-UV and MALS detection and separation of the total cell lysate; fractionation of the proteome according to the MW, data processing and interpretation. For this protocol, we run eight fractionations and pull each fraction to ensure the necessary amount of protein for native-PAGE and proteomic analysis (data not shown). Pooled fractions are dialyzed (to reduce the amount of salt) and lyophilized. Native-PAGE confirms protein separation according to MW and increased amounts of HMW protein aggregates in the PQ-treated sample as compared with the control (CTR).
- iv. *Fractionation and characterization of the total cell lysates under mild denaturing conditions:* The purpose of this stage is to determine the biophysical nature of the PQ-induced protein aggregates, especially the irreversible urea-resistant covalent HMW protein aggregates. The experimental setup includes HF5-UV and MALS detection and separation of the total cell lysate, fractionation of the proteome according to the MW, data processing and interpretation. The MW ranges used to fractionate the proteome is identical to the ones used under native conditions. This provides a simple way to compare protein aggregates under native and mild denaturing conditions. Changes in MW, hydrodynamic and r.m.s. radius, as well as protein conformation, are observed when comparing the results obtained under different separation conditions.

Advantages and limitations of the protocol

Given the size of the separation device and the calculated amount of HMW protein aggregates present in the total lysates (approximated at 2–8%), fraction pooling from different runs is required in order to obtain the necessary amount of protein for further analysis (SDS-PAGE and MS). The protocol time can be reduced considerably if further complementary analyses of the separated protein aggregates are not required. On-line coupling of HF5 with UV and MALS detection is sensitive enough to fully characterize cell lysate amounts down to 20 µg of total protein (hydrodynamic size, absolute MW, r.m.s. radius, molecular conformation and relative quantification of HMW protein aggregates as the percentage of the total protein).

However, when used for semipreparative purposes, there are risks of overloading the HF5 device (Fig. 2a–c) if the total protein amount exceeds 100–150 µg. The commercial HF5

cartridge (Fig. 1) overloads at ~50 μ g of total protein amount. Aside from the double-length HF5 device used in this protocol, possible scale-up of the separation device may include the use of multiple HF5 commercial cartridges working in parallel, as described by Lee *et al.*⁵².

Comparison with other methods

Building on the increased interest in analyzing protein aggregates, a wide variety of analytical methods have been used to separate the aggregates, including filter assays, sedimentation velocity analytical ultracentrifugation (SV-AUC), gel electrophoresis, size-exclusion chromatography (SEC) and its macro-column variant fast protein liquid chromatography (FPLC)^{57–61}. SV-AUC can be used for proteome samples dispersed in different media, over a wide size range of protein aggregates. Nevertheless, the analyses cannot resolve closely sized aggregates, it is time-consuming and it has low sample throughput⁶². SEC and, by extension, FPLC are also often used to separate protein aggregates. However, protein aggregates often adsorb to the column matrix, large protein aggregates can be lost in the void volume and both techniques fail to resolve aggregates over a large dynamic range⁶³. In addition, all the above-mentioned techniques cannot be performed in high-detergent concentrations or under denaturing conditions, as used to distinguish between covalent versus non-covalent protein aggregates. Finally, gel electrophoresis, which is also commonly used to separate protein aggregates, does not provide an accurate MW analysis, nor does it provide any information on the biophysical properties of the aggregates.

Therefore, an analytical methodology that is capable of accurately separating protein aggregates on the basis of their molecular size and their biophysical properties would prove to be extremely useful in the characterization of physiological and pathological protein aggregation.

The separation principle of HF5

HF5 relies on differences in diffusion coefficient (D)—which is specific to each protein and correlated with protein size (hydrodynamic radius, r_h) and MW⁴⁵. When proteome samples are injected into the hollow fiber, they are first subjected to the action of two longitudinal flows entering from both ends of the separation device, thus having opposite directions with a typical inlet/outlet flow ratio of 20:80 (Fig. 1a). This results in the sample being concentrated in a narrow band at the meeting point of the two streams (where the resulting longitudinal flow is null), called the focus position (Fig. 1a). The proteome components are then subjected to two types of forces: the radial field, which moves them toward the hollow fiber wall (black arrows in Fig. 1b), and the protein diffusion force, which moves them toward the hollow fiber center (white arrows in Fig. 1b). The radial field force will reach equilibrium with the protein diffusive force (steady state), at a distance from the channel wall that is specific to each diffusion coefficient (D) and thus unique to each protein.

At this point, the outlet flow is switched off, and thus the inlet flow will direct the proteome components toward the channel exit, whereas the combination of the radial field force and diffusion force will maintain each protein at the specific distance from the channel wall that was previously reached at steady state (Fig. 1b). Smaller proteins with a larger diffusion

coefficient will be the first to elute, followed by the other proteome components in increasing size order.

MATERIALS

REAGENTS

- Acetic acid (Fisher Scientific, cat. no. A35-500)
- Ethanol, molecular biology grade (Fisher Scientific, cat. no. BP2818-4)
- Methanol (Fisher Scientific, cat. no. A412-1)
- β -Mercaptoethanol (Sigma-Aldrich, cat. no. M6250) ! **CAUTION** This material is toxic, and it should be handled in a fume hood; use appropriate personal protective equipment.
- Glycerol (Fisher Scientific, cat. no. BP229-1)
- Bromophenol blue (Bio-Rad, cat. no. 161-0404)
- SDS, 10% (wt/vol) solution for electrophoresis (Fisher Scientific, cat. no. BP2436-1)
- DTT (Sigma-Aldrich, cat. no. D0632)
- NaCl (Sigma-Aldrich, cat. no. S9888)
- BSA (Sigma-Aldrich, cat. no. A9418)
- Urea (Sigma-Aldrich, cat. no. U5378)
- PBS, 10 $\times$ (Sigma-Aldrich, cat. no. P5493-1L)
- Tris-HCl, 1.5 M, pH 8.8 (Teknova, cat. no. T1588)
- Triton X-100 for molecular biology (Sigma-Aldrich, cat. no. T8787)
- Complete protease inhibitor cocktail (Sigma-Aldrich, cat. no. P8340)
- Complete medium HyClone DMEM/high glucose (Thermo Scientific, cat. no. SH30022.01)
- FBS, 10% (vol/vol) (GE Healthcare, cat. no. SH30071.01)
- HEPES buffer, 1% (Fisher Scientific, cat. no. BP299-100)
- Minimum essential medium (MEM) non-essential amino acids (Sigma-Aldrich, cat. no. M7145)
- Sodium pyruvate, 1 mM (MP Biomedicals, cat. no. 0916820)
- Penicillin, 10 U/ml and streptomycin, 100 μ g/ml (Sigma-Aldrich, cat. no. P4333)
- *N,N'*-dimethyl-4,4'-bipyridinium dichloride/methyl viologen dichloride hydrate (Paraquat dichloride, PQ; Sigma-Aldrich, cat. no. 856177) ! **CAUTION** This material is toxic, and it should be handled in a fume hood; use appropriate personal protective equipment.

- Jaws II cells (American Type Culture Collection, cat. no. CLR-11904), a semiadherent dendritic cell (DC) line established from C57BL/6 bone marrow. Even though the method was established using Jaws cells, we also tested primary DCs. We do believe that the fractionation can be performed on different cell lysates, albeit the dose of PQ used to induce aggregation should be titrated in different cell types
- Pierce universal nuclease for cell lysis (Thermo Scientific, Pierce, cat. no. 88700)
- Protein markers mixture for native PAGE, such as NativeMark unstained protein standard, Novex (Life Technologies, cat. no. LC0725)
- Bicinchoninic acid (BCA) assay kit, such as Pierce BCA protein assay kit (Pierce, cat. no. 23227)
- Spectrophotometric assay kit, such as OxiSelect protein carbonyl spectrophotometric assay kit (Cell Biolabs, cat. no. STA-315)
- Silver stain kit, such as Pierce color silver stain kit (Pierce, cat. no. 24597)
- SDS-PAGE gel, such as Mini-PROTEAN TGX precast gel (4–15%, wt/vol) with a MW range of 20–250 kDa (Bio-Rad, cat. no. 456-1089)

Other reagents (necessary only for additional analyses after fractionation, on the protein fractions)

- Native PAGE gel, such as NativePAGE Novex Bis-Tris gel system (3–12%, wt/vol) with a MW range of 30–10,000 kDa (Life Technologies, cat. no. BN1001BOX)

EQUIPMENT

Consumables

- Polyethersulfone (PES) hollow fibers (Microdyn-Nadir, FK 20, cat. no. FUS 0181: 1,066-mm-long modules, 0.8-mm inner diameter, 1.3-mm outer diameter and 10-kDa MWCO, corresponding to an average pore diameter of 5 nm). The 1,066-mm-long modules provide the hollow fiber required for two or three cartridges (340-mm long; in this protocol, used for semipreparative purposes). For the separation and characterization of lower amounts of protein (<50 µg of total protein), disposable HF5 commercial cartridges are the optimal choice (Superon, distributed by Wyatt Technology Europe)
- Falcon tubes, 50 ml (Becton Dickinson, cat. no. 352070)
- Eppendorf tubes (Fisher Scientific, cat. no. 022363204)
- Steritop filtering units, 0.22-µm pore membrane for carrier solution (Millipore, filtering units: cat. no. SCGPT05RE (500 ml) or cat. no. SCGPT10RE (1,000 ml), and bottles for collection and storage: cat. no. SC00B05RE (500 ml) or cat. no. SC00B10RE (1,000 ml))

- Syringe filters, 0.45- μ m pore membrane for cell lysate samples (Millex-HV from Millipore, cat. no. slhv033rs)
- SnakeSkin pleated dialysis tubing (Pierce, cat. no. 88243)
- HPLC vials, such as conical-bottom polypropylene screw-top vials (Supelco vials from Sigma-Aldrich, cat. no. 27410 or from Agilent Technologies, cat. no. 5190-2243)

Instrumentation

- HPLC system (such as Agilent 1100 series, Agilent Technologies) consisting of: autosampler (Agilent 1100); degasser (Agilent 1100); quaternary pump (Agilent 1100, although an isocratic pump should suffice); diode array detector (DAD; Agilent 1100, although a variable wavelength detector set at 280 nm should suffice); ChemStation software for Agilent 1100 series (Agilent Technologies)
- Eclipse DUALTEC FFF separation system (Superon, distributed by Wyatt Technology Europe)
- Eclipse HF5 cartridges: two commercial chasings connected through a union piece (Superon, distributed by Wyatt Technology Europe)
- Eclipse plug-in for ChemStation software (Superon)
- ISIS simulation software (Superon)
- Multiangle light-scattering (MALS) detector, such as DAWN EOS (Wyatt Technology)
- Astra software (Wyatt Technology)
- Spectrophotometer for BCA assay and carbonyl assay such as SmartSpec 3000 Spectrophotometer (Bio-Rad, cat. no. 170-2501)
- Elix 3 UV water purification system (Millipore) for Milli-Q water
- Lyophilizer (SP scientific, BT2KXL, cat. no. 218577)
- Tabletop centrifuge (Continental Lab Products, silent spin)
- Centrifuge (Sorvall legend RT)
- Heating block
- Electrophoresis system (Bio-Rad, cat. no. 153BR 26007)
- CO₂ incubator (Heracell 150)

Software specification

- ChemStation data system for Agilent instrumentation version B.04.02 (Agilent Technologies)
- Eclipse plug-in for ChemStation, Wyatt Eclipse ChemStation version 3.5.02 (Superon)

- ISIS simulation software version 1.2.0 (206; Superon)
- Astra software version 6.0.6 (Wyatt Technology)
- Operating system: Microsoft Windows XP or higher (please contact Superon for exact requirements)
- ExPASy ProtParam free bioinformatics tool (<http://www.expasy.org/>)

REAGENT SETUP

Cell culture medium—Prepare the cell culture medium by adding 10% (vol/vol) FBS, 1% (vol/vol) HEPES buffer, MEM non-essential amino acids, 1 mM sodium pyruvate, 10 U/ml penicillin and 100 µg/ml streptomycin to complete medium HyClone DMEM/high glucose. Medium can be stored at 4 °C for up to 1 month.

PQ solutions required to induce oxidative stress on cells—Prepare *N,N'*-dimethyl-4,4'-bipyridinium dichloride (PQ) solutions at concentrations of 0.25, 5 and 10 mM, by adding the appropriate amounts of PQ in PBS. PQ-containing medium should be freshly prepared. ! **CAUTION** When you are using PQ, use appropriate protection, because it is very toxic.

Lysis buffer—Prepare the cell lysis buffer with the following composition: 150 mM NaCl, 50 mM Tris-HCl, 1% (vol/vol) Nonidet P-40, 10 mM DTT and 5 mM EDTA. Supplement it upon use with 1× protease inhibitor cocktail. ▲ **CRITICAL** Keep the stock solution of the lysis buffer at 4 °C for no longer than 1 month, and add protease inhibitors freshly before use.

HF5 carrier solutions—Native carrier solution (50 mM Tris-HCl supplemented with 150 mM NaCl): add 8.775 g of NaCl to 33 ml of Tris-HCl 1.5 M solution, and fill it with Milli-Q water up to 1 liter. Check the pH (8.0) and filter the solution on 0.22-µm (Steritop Millipore) filter membranes. ! **CAUTION** Always filter the carrier solution. Prepare 1,000 ml at a time and store it at 4 °C. Replace the solution at least once a week.

Mild denaturing carrier solution (50 mM Tris-HCl supplemented with 150 mM NaCl and 2 M urea): add 120.12 g of urea and 8.775 g of NaCl to 33 ml of Tris-HCl 1.5 M solution, and fill it with Milli-Q water up to 1 liter. Check the pH (8.0) and filter the solution on 0.22-µm (Steritop Millipore) filter membranes. ! **CAUTION** Always filter the carrier solution. Prepare 500 ml at a time, and store it at room temperature (24 °C). Replace the solution at least once a week.

BSA standard solution—Dissolve 1 mg of BSA in 1 ml of Milli-Q water to obtain a 0.1% (wt/vol) BSA solution. This standard will be used to verify periodically the proper functioning of the separation-detection system. Significant shifts in the BSA retention time or abnormal (very intense) UV and/or light-scattering peaks at the end of the run usually indicate the depletion of the hollow fiber (which will need replacing). The BSA standard's retention time is also used to correct the time delay between detectors and band broadening.

BSA is a control protein with known MW, as well as an isotropic scatterer that is used to normalize or correct the intensity of the MALS detectors (diodes at 18 different angles).

Cleaning solution for the separation-detection (HPLC-FFF-MALS) system—

Prepare a 0.2% (vol/vol) solution of Triton X-100 by adding 1 ml of Triton X-100 in 500 ml of purified water (Milli-Q water). This volume should suffice for an overnight cleaning of the separation and detection system (10 h of cleaning at 0.5 ml/min).

Dialysis buffer (10 mM Tris-Cl)—Measure 6.67 ml of 1.5 M Tris-HCl and fill it with Milli-Q water up to 1 liter. Prepare at least 4 liters of dialysis buffer per shift. If the carrier solution (for the HF5 separation) contains urea, replace the dialysis buffer every 6 h.

EQUIPMENT SETUP

Overview of the HF5-UV-MALS instrument setup—This setup includes typical HPLC components (degasser, pump, autosampler and DAD), as well as the FFF separation module (Eclipse DUALTEC), HF5 separation device and a MALS detector (DAWN EOS; Fig. 2). We recommend degassing the carrier solution to avoid false peaks in the UV and light-scattering signals. The pump flow rate is controlled through the Eclipse plug-in for ChemStation; the same plug-in is used to generate the flows inside the Eclipse DUALTEC module—flows that are necessary to perform the HF5 separation.

An autosampler delivers the sample into the HF5 device (Fig. 1a,b). The sample enters the DAD, which sends the UV signals variation against elution time to the PC through the ChemStation software (for proteins, typically set at 215, 260 and 280 nm). Finally, the sample enters the MALS detector, which sends the UV signal (for proteins, set at 280 nm) and the light-scattering signal from all 18 angles to the PC through the Astra software. Once the HF5 run is completed, the data can be processed. The Astra software is used to calculate the sample concentration, absolute MW and the r.m.s. radius against the elution time. It is also used to determine the molecular conformation of the sample components. ISIS software is used to calculate the hydrodynamic radius from the retention time value. In highly complex samples, the hydrodynamic radius can be only estimated.

HPLC and FFF module setup—Make the necessary connections (tubing and cables) between the Eclipse DUALTEC FFF separation system, the HPLC components and the PC. Follow the instructions provided by the manufacturer (Superon, distributed by Wyatt Technology Europe) for ChemStation software and Eclipse plug-in installation, instrument setup and operation. Use the ChemStation chromatographic software to control the HPLC system components (autosampler and DAD), to monitor the separation and for data processing. Set the UV lamp (DAD setup) wavelengths at 280, 215 and 260 nm, which are necessary for protein detection. Use the Eclipse plug-in for ChemStation (Superon) to operate the FFF separation module according to the manufacturer's instructions. Use the ISIS software using the instructions provided with the software for FFF method simulation and optimization and for the determination of the hydrodynamic size of the proteins and protein aggregates from their specific retention times.

HF5 device setup—The HF5 commercial cartridge assembly and the modes of operation of the Eclipse DUALTEC system have already been previously described in recent literature⁸ and in the Eclipse DUALTEC instructions manual. The protocol presented in this paper entails using a longer HF5 separation device (scale-up for semipreparative purposes), which is assembled by joining together two 17-cm-long commercial cartridges with a cap nut, resulting in a 34-cm-long channel ($2 \times 17 \text{ cm} = 34 \text{ cm}$)—the double-length HF5 device (Fig. 2a–c). Once the chasings are connected, insert a single hollow fiber membrane (34 cm long) and seal it by tightening the cap nuts at both ends (described in detail by Johann *et al.*⁴⁷). By using the double-length HF5 device, an amount of ~100–150 µg of proteome can be loaded and separated under optimal conditions.

Attach the double-length HF5 device to port A on the Eclipse DUALTEC separation system according to the manufacturer's instructions, by paying attention to possible leaks. By using the Eclipse plug-in for ChemStation HPLC software, set the detector flow rate at 0.5–0.6 ml/min in Elution mode and flush the separation system (HF5-UV-MALS) with carrier solution for 1.5–2 h. This procedure, called 'system conditioning', eliminates the air from the HF5 cartridge and helps in obtaining stable UV and light-scattering baselines.

Perform a hollow fiber conditioning run (BSA make-up run), according to the instructions provided in the Eclipse DUALTEC operation manual, by loading the appropriate HF5 method for BSA separation (found among the preset methods) and by injecting 20–30 µg of BSA standard sample (20–30 µl of 0.1% (wt/vol) standard BSA solution). This procedure is required each time the hollow fiber is replaced with a new one.

Eclipse method setup for HF5—Follow the instructions provided in the Eclipse manual to set up the Eclipse HF5 method for separation of total cell lysates, which uses the device depicted in Figure 2a–c. The HF5 method parameters used in this protocol, to be set in the Eclipse method dialog box, are reported in Table 1. Save the method and reload it before separating the cell lysates. Remember to save the method again if changes are made and to reload it before data acquisition. Do not save the method if the only parameter changing between data acquisitions is the sample volume. ▲ **CRITICAL** Subtract the focus and focus injection duration time from the retention time value that appears on the ChemStation report at the end of the run to obtain the true sample retention time. The corrected retention time is to be used in the ISIS calculations.

MALS detector setup—Make the necessary connections (tubing and cables) between the MALS detector (DAWN EOS) and the DAD, the Eclipse DUALTEC and the PC. Please refer to Astra instructions manual for instrument configuration, Astra software installation and operation.

Astra method setup—Follow the instructions provided by the manufacturer for the creation of new Astra methods. Create a new Astra method for BSA separation by setting the appropriate flow rates (0.35 ml/min). Check that the Astra method starts automatically when the HF5 separation starts and that the duration of the Astra method and the Eclipse method match. Perform a BSA separation and process the data (UV and light scattering) according to the manufacturer's instructions regarding baseline setting, normalization,

detector delay and band broadening. The calculated MW of the BSA monomer should match the value declared on the product sheet (~67 kDa). The BSA extinction coefficient at 280 nm ($\epsilon_{280\text{ nm}} 0.1\%$) used in the MW computation is 0.667 (ml/mg $\times$ cm). Create a new Astra method for the separation of cell lysates (flow rate: 0.5 ml/min) and import the normalization coefficients from the previous BSA run. Save the method.

Spectrophotometer: calibration curve with BSA solutions—Prepare the necessary dilutions using the BSA standard provided in the kit. Follow the manufacturer's instructions provided with the BCA assay kit to measure the BSA absorbance. Prepare a standard curve by plotting the average blank- corrected 562-nm measurement for each BSA standard versus its concentration in $\mu\text{g/ml}$. The standard curve will be used to determine the protein concentration of each cell lysate.

PROCEDURE

Cell culture ● TIMING 24 h

1| Culture Jaws II cells (CLR-11904; American Type Culture Collection) in complete medium HyClone DMEM/high glucose supplemented with 10% FBS and 1% HEPES buffer (vol/vol), MEM non-essential amino acids, 1 mM sodium pyruvate, 10 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin.

▲ **CRITICAL STEP** Use enough cells to retrieve 50 μg of total protein lysate for each run that you plan to perform. In our hands, two million cells are needed to retrieve 100 μg .

PQ treatment ● TIMING 6 h

2| Add PQ to the cells at concentrations of 0.25, 5 and 10 mM (20 million cells for each condition, in triplicate) to induce oxidative stress. Incubate PQ-treated and untreated cells at 37 °C and 5% CO₂ for 12 h. Untreated cells serve as negative controls. The protein carbonyl spectrophotometric assay (Step 12) will verify whether the PQ treatment worked.

! **CAUTION** Take necessary precautions (i.e., work under a fume hood) when handling PQ solutions.

3| Collect the CTR (untreated cells) and PQ-treated cells in 50-ml Falcon tubes, and centrifuge them at 220g for 6 min at room temperature.

4| Wash the cells three times with sterile PBS and discard the supernatant, remembering to transfer them to Eppendorf tubes before the last wash.

Cell lysis ● TIMING 1 h

5| Add 150–250 μl of lysis buffer freshly supplemented with 1 $\times$ protease inhibitor cocktail to the cellular pellet and incubate the mixture on ice for 40 min.

6| Centrifuge the mixture at 11,750g for 30 min at 4 °C. The cellular pellet will be visible at the bottom of the tubes.

7| Carefully collect the supernatant for further HF5 analysis.

Protein purification by enzymatic digestion with universal nuclease ● TIMING 30–40 min

8| Add universal nuclease (0.1 μ l/1 ml of lysate or prepare a 1:100 dilution and add 10 μ l/1 ml cell lysate) to process nucleic acids that would interfere with protein separation, and incubate the sample on ice for 30 min.

▲ **CRITICAL STEP** The universal nuclease, similar to benzonase nuclease, has much higher specificity for degrading nucleic acids (RNA and DNA: single-stranded, double-stranded, linear or circular) compared with DNase, and therefore it is the ideal choice when complete removal of nucleic acids is required during the preparation of cell lysates.

9| Filter the lysate samples on 0.45- μ m syringe (sterile) filters.

▲ **CRITICAL STEP** It is highly important to filter the purified cell lysates, not only to eliminate the digested DNA and RNA but also to avoid blocking the tubing and/or the flow cells of the detectors with large particulates during HF5-UV-MALS separation-characterization.

▲ **PAUSE POINT** The purified cell lysates can be stored at -80°C for 1 month or at -20°C for ~1 week. Prolonged storage increases protein carbonylation and aggregation.

Protein quantification ● TIMING 2 h total (0.5 h per sample for four samples)

10| Quantify the protein amount against a BSA standard curve, using the Pierce BCA protein assay kit according to the manufacturer's instructions. The protein amount deriving from 20 million cells should be in the concentration range of 5–10 mg/ml.

11| Prepare 150–200- μ l aliquots of each sample.

■ **PAUSE POINT** Store the aliquots at -80°C . Thaw one aliquot at a time and store it at 4°C between analyses (maximum 12 h).

Protein carbonyl spectrophotometric assay to confirm the effectiveness of the PQ treatment ● TIMING 1 h

12| Determine the total protein carbonyl content in the CTR and PQ-treated cell lysates spectrophotometrically by reading the absorbance of the 2,4-dinitrophenylhydrazine (DNPH)-derivatized carbonyl groups at 375 nm, according to the manufacturer's instructions provided with the OxiSelect protein carbonyl spectrophotometric assay kit.

13| Normalize the results to the amount of total protein in each sample. An example is depicted in Figure 3a, which shows an increased amount of protein carbonyl groups proportional to the PQ molarity. This assay proves that the PQ treatment induces protein modifications (carbonylation), which are known to lead to aggregation during aging.

▲ **CRITICAL STEP** If you do not see an increase in protein carbonylation, the PQ treatment did not work.

? TROUBLESHOOTING

SDS-PAGE on total cell lysates to confirm the presence of protein aggregates induced by PQ treatment ● TIMING 3 h

14| Prepare the SDS-PAGE sample buffer containing 62.5 mM Tris-Cl, pH 6.8, 2% (wt/vol) SDS, 25% (vol/vol) glycerol and 0.01% (vol/vol) bromophenol blue.

15| Dilute 20 µg of total cell lysates in sample buffer, freshly supplemented with 2-mercaptoethanol, and incubate the mixture for 5 min at 110 °C.

16| Prepare the Tris/glycine/SDS running buffer containing 25 mM Tris, 192 mM glycine and 0.1% (wt/vol) SDS, and adjust the pH to 8.3.

17| Load the diluted lysate samples on a Mini-PROTEAN TGX gel (4–15% (wt/vol)), recommended for the MW range of 20–250 kDa.

18| Perform the electrophoretic run in Tris/glycine/SDS running buffer for 1.5 h.

19| Re-equilibrate the gel in water for 5 min, and stain it by using the Pierce color silver stain kit (according to the procedure reported in the kit). An example of an SDS-PAGE gel performed on CTR and PQ-treated samples is reported in Figure 3b. The gel shows higher amounts of HMW protein aggregates in the PQ-treated samples as compared with the CTR sample, proportional to the PQ molarity. The gel also shows the presence of low-molecular-weight proteins (oxidative stress-related cleavage products).

▲ CRITICAL STEP Make sure that the purified lysate samples have been filtered (0.450-µm sterile filters) in order to avoid blocking the MALS detector flow cell and the HPLC tubing with large particulates.

Checking the HF5-UV-MALS system with a standard protein mixture ● TIMING 2 h total

20| Load the Eclipse (HF5) method for cell lysates (Equipment Setup), inject 5 µl of the NativeMark unstained protein standard and perform the HF5 separation. The same protein markers are used to perform the native-PAGE. ChemStation and Astra acquisitions should start simultaneously. Save the Astra file at the end of the run (the ChemStation file is saved automatically).

? TROUBLESHOOTING

21| Use the ExPASy ProtParam free bioinformatics tool (<http://www.expasy.org/>) to compute the specific extinction coefficient ($\epsilon_{280\text{ nm}}$ 0.1%) for all protein standards in the mixture, and enter these values in the Astra file.

22| Assign the peak retention times to the protein species in the mixture and calculate their MW according to the Astra manual instructions for data processing. An example is shown in Figure 4, in which the correlation between the native marker MW and their retention time, separated using the cell lysates method, is reported through red empty circles. Their elution order is as follows: soybean trypsin inhibitor, 20 kDa; BSA, 66 kDa; lactate dehydrogenase, 146 kDa; β -phycoerythrin, 242 kDa; apoferritin band 1, 480 kDa; apoferritin band 2, 720 kDa; (IgM pentamer, 1,048 kDa; and IgM hexamer, 1,236 kDa.

Analyze the NativeMark unstained protein standard mixture from time to time (i.e., once a week) and compare the retention time values.

? TROUBLESHOOTING

23| (Optional) Use ISIS simulation software to predict the (theoretical) retention time, hydrodynamic size and diffusion coefficient values by inputting the EclipseHF5 method flow rates and the known MW values (declared by Life Technologies in the product sheet).

? TROUBLESHOOTING

Cell lysate fractionation under native conditions: HF5-UV-MALS ● TIMING 6 h total

24| Inject the purified and filtered cell lysate and perform the HF5 separation (Fig. 4a).

▲ **CRITICAL STEP** Start with the CTR (untreated) sample, and then inject the PQ-treated cell lysates in increasing PQ concentrations in order to avoid contamination. Make sure not to overload the HF5 device; it has a maximum capacity of 100–150 µg of total protein.

25| Fractionate the lysate samples in sequence and save the Astra files as soon as the individual runs are completed (the ChemStation files are saved automatically).

■ **PAUSE POINT** Data processing can be performed at a later time point.

Data processing and interpretation: ISIS and Astra cell lysate characterization under native conditions ● TIMING 2–3 h total

26| Use ISIS to calculate/estimate the hydrodynamic size of proteins or protein aggregates in the total cell lysates on the basis of their experimental retention times. The viscosity values for the carrier solutions are extrapolated from data found in literature⁶⁴ ($\eta_{\text{water}, 25\text{ }^{\circ}\text{C}} = 10\text{--}3\text{ N}\cdot\text{s}/\text{m}^2$ for Tris-HCl buffer and $1.0909 \times \eta_{\text{water}, 25\text{ }^{\circ}\text{C}}$ for Tris-HCl + 2 M urea).

An example is depicted in Figure 4a, in which the ISIS-derived hydrodynamic radius values were plotted as the bottom *x*-axis. These values serve as a reference when comparing results obtained under different experimental conditions.

27| Calculate the MW range of the cell lysate from the light-scattering intensity registered by detectors 2 through 18 and the concentration signal from the UV detector (280 nm) correlated through a first-degree Zimm model, according to the instructions provided in the Astra manual. Set an average value of 1.0 (ml/mg × cm) for the extinction coefficient at 280 nm ($\epsilon_{280\text{ nm}}^{0.1\%}$) in all MW calculations for the cell lysates in the Peaks tab. The peak or selection chosen for MW range calculation should start after the void peak and end with the run (it can be reduced later to display only the MW range of interest, as in Fig. 4a). Open EasyGraph and choose ‘molar mass’ to display the calculated MW range.

? TROUBLESHOOTING

28| Use EasyGraph and choose ‘RMS radius’ to calculate the r.m.s. radius range for the cell lysate sample from the angular dependency of the scattered light correlated through a first-degree Zimm model. The results will be displayed on the screen (Fig. 4b).

? TROUBLESHOOTING

29| Use EasyGraph and choose ‘RMS conformation plot’ to determine the molecular conformation of the protein aggregates. The correlation plots (conformation plots) are generated from experimental data, by plotting the r.m.s. radius values against the corresponding MW values (both in logarithmic scale). The plots are fitted linearly and the slope values are assigned to known conformation types. A slope value of 1.0 corresponds to a rod conformation, a slope value of 0.5–0.6 corresponds to a random coil conformation and a slope value of 0.33 corresponds to a sphere conformation. The trend (slope and/or change of the slope) of the molecular conformations is determined for r.m.s. radius values of >10 nm (owing to DAWN EOS instrumental range limitation), with corresponding MW values usually $>1 \times 10^6$ g/mol.

Examples of correlation plots are shown in Figure 4b, and they show molecular conformation changes (the protein aggregates become more compact) caused by the PQ treatment. These conformational changes are very likely due to protein carbonylation, which is reported in Figure 3a. Moreover, the correlation plots show the presence of HMW species ($>3.5 \times 10^6$ Da) only in the PQ-treated samples (also observed in Figs. 3b and 4d).

? TROUBLESHOOTING

30| Calculate the relative protein aggregate amounts, as % relative to the total protein in the lysates (Fig. 4a; Table 2). Choose *Results* → *Report (summary)* and scroll down to Peak Results—Mass fraction (%).

? TROUBLESHOOTING

Create virtual protein fractions by subdividing the chromatographic space ● TIMING 1–1.5 h

31| Load the Astra acquisition files (CTR and PQ-treated samples) and subdivide the MW range into fractions by selecting the MW of interest. Set narrower fractions for (high-abundance) smaller proteins (fractions F1–F3 in Fig. 4c, which elute in the first 15 min) and larger fractions for (low-abundance) large proteins and protein aggregates (fractions F4–F6 in Fig. 4c).

An example is shown in Table 3, in which we report the complete characterization of the virtual protein fractions selected for the purpose of this protocol. All the data reported in Table 3 were retrieved from the Astra Report, on the basis of the virtual fraction selections in EasyGraph, and from the hydrodynamic radius values obtained from ISIS.

▲ CRITICAL STEP Use the same settings for all Astra files (fraction start and end) to effectively establish differences between the untreated and PQ-treated lysate samples.

? TROUBLESHOOTING

Collect and pool the protein fractions for complementary analyses ● TIMING 4 d total

32| Once the virtual fraction selection is adjusted to your needs (example shown in Fig. 4c), proceed to actual protein fraction collection for the CTR and the lysate sample treated with the highest concentration of PQ based on the timetable of fractions established at Step 29.

▲ **CRITICAL STEP** Given the reduced size of the HF5 device (Fig. 2a–c) and the low-abundance HMW protein aggregates in the cell lysates (Tables 2 and 3), combine the fractions from eight independent runs (CTR and 10 mM PQ samples).

▲ **CRITICAL STEP** Inject as much as 100–150 µg of total cell lysate for each run. Perform four or five fraction pools and clean the system by flushing the cleaning solution overnight, followed by flushing filtered water for at least 2–3 h the next morning. Once the system is clean, replace the hollow fiber and connect it to the FFF module. The PES hollow fiber will be depleted quickly after four or five runs. Perform the fiber make-up procedure with the BSA standard, according to the Eclipse instructions manual.

33| Monitor the separation process on the PC screen and save the acquisition files. Data processing is not necessary at this point.

■ **PAUSE POINT** Store the protein fractions in Falcon tubes at 4 °C overnight. Proceed with fraction dialysis as soon as possible.

Protein fraction dialysis ● TIMING 12 h + storage time

▲ **CRITICAL** The proteins are eluted at 0.5 ml/min during the separation process. Consequently, when the fraction pooling is completed, the narrowest fraction (F1, 2.5 min) will contain high-abundance proteins dispersed in 10 ml of native carrier solution, whereas the largest fractions (F4–F6) will contain low-abundance protein aggregates dispersed in 40 ml of carrier solution.

34| Load the protein fractions into Skin pleated dialysis tubing of appropriate length (MWCO: 3,500 Da, capacity of 3.7 ml/cm). Divide the large protein fractions (F4–F6, 40 ml) and load them into two dialysis tubes.

35| Place the loaded dialysis tubes into large containers filled with 4 liters of dialysis buffer (10 mM Tris-HCl solution at pH 8.0).

36| Dialyze overnight in order to remove the NaCl concentration.

▲ **CRITICAL STEP** Upon dialysis completion, transfer the protein fractions into Falcon tubes and freeze them at –80 °C to speed up the lyophilization process.

Protein fraction lyophilization ● TIMING 12–24 h

▲ **CRITICAL** The lyophilization of the CTR sample fractions should begin immediately after their dialysis and storage at –80 °C, on day 3 of fractions pooling, to optimize the experimental time.

37| Place the (frozen) protein fractions into the lyophilizer.

Complementary analyses: native PAGE on the protein fractions ● TIMING 5 h total

38| Resuspend the lyophilized proteins in a total volume of 100 µl of Milli-Q water.

39| Quantify the protein amount in each fraction by BCA assay (as in Step 10).

40| Normalize the protein concentration by resuspending in native PAGE sample buffer (4×) and cathode buffer additive (Invitrogen) according to the manufacturer's instructions.

41| Load the same amount of proteins for each fraction on a NativePAGE Novex Bis-Tris gel system (3–12%, wt/vol).

42| Run the gel at 150 V for 110 min according to the supplier's instructions.

43| Upon completion of electrophoresis, re-equilibrate the gel in water for 5 min and stain it using the Pierce color silver stain kit, according to the manufacturer's instructions. An example of native-PAGE results is shown in Figure 4d, confirming protein separation by HF5 according to MW and hydrodynamic size, detected as the increment in MW in progressive fractions. The gel shows the presence of very HMW aggregates in fraction 6 of PQ-treated samples but not in the CTR lysate, which is in agreement with the HF5-UV-MALS results (Fig. 4a).

■ **PAUSE POINT** We used native-PAGE on the protein fractions separated by HF5 to prove the suitability of the HF5 method to separate and characterize protein aggregates of different MWs from total cell lysates, as HF5-UV-MALS is not a routine technique for proteome prefractionation. The protein aggregates present in the native-PAGE lanes can be further processed by mass spectrometry for proteomics studies.

Determination of the biophysical nature of PQ-induced HMW protein aggregates: HF5-UV-MALS ● TIMING 4–4.5 h

44| Perform the separation in a carrier solution containing a denaturant (urea) to discern noncovalent (reversible) from covalent aggregates (urea-resistant/irreversible) after Schiff base formation.

45| Repeat the operations described in Steps 24–31 for data acquisition, processing and interpretation, but use the mild denaturing carrier solution instead of the native one. Perform the separations only on the CTR sample and the sample treated with the highest concentration of PQ (10 mM PQ)—the two extremes in our experimental design.

? TROUBLESHOOTING

Step 13: In our experience, not all the batches of PQ work the same. Always compare new batches of PQ with previously used ones by adding the same concentration to the cultured cells and by comparing the amounts of protein carbonylation (Step 12).

Further troubleshooting advice can be found in Table 4.

● TIMING

Step 1, cell culture: 24 h

Steps 2–4, PQ treatment: 6 h

Steps 5–7, cell lysis: 1 h

Steps 8 and 9, protein purification by enzymatic digestion with universal nuclease: 30–40 min

Steps 10 and 11, protein quantification: 2 h total (0.5 h per sample for four samples)

Steps 12 and 13, protein carbonyl spectrophotometric assay to confirm the effectiveness of the PQ treatment (protein carbonylation, proportional to the PQ molarity): 1 h

Steps 14–19, SDS-PAGE on total cell lysates to confirm the presence of protein aggregates induced by the PQ treatment: 3 h Steps

Steps 20–23, checking the proper functioning of the HF5-UV-MALS system using a standard protein mixture: 2 h total (1.5 h for data acquisition and 30–45 min for data processing)

Steps 24 and 25, cell lysate fractionation under native conditions: HF5-UV-MALS: 6 h total (1.5 h for data acquisition per sample for four samples)

Steps 26–30, data processing and interpretation: ISIS and Astra cell lysate characterization under native conditions: 2–3 h total (30–45 min per sample for four samples)

Step 31, create virtual protein fractions by subdividing the chromatographic space: 1–1.5 h

Steps 32 and 33, collect and pool the protein fractions for complementary analyses: 4 d total (2 d per sample for two samples, with an average of four or five pools per day)

Steps 34–36, protein fraction dialysis: 12 h + storage time

Step 37, protein fraction lyophilization: 12–24 h (depending on the volume)

Steps 38–43, complementary analyses: native PAGE on the protein fractions: 5 h total (3 h for protein quantification by BCA assay and 2 h for electrophoresis)

Steps 44 and 45 (repeat Steps 24–31), determination of the biophysical nature of PQ-induced HMW protein aggregates: HF5-UV-MALS: 4–4.5 h (1.5 h per sample for data acquisition for four 4 samples, and 30–45 min per sample for data processing for four samples): 4–4.5 h

ANTICIPATED RESULTS

With this protocol, the HF5-UV-MALS setup successfully analyzes and fractionates protein aggregates, as found in total cell lysates. The experimental design is set up using PQ-induced carbonylation, which causes the formation of HMW protein aggregates that closely resemble the ones observed in aging and chronic inflammatory or degenerative conditions. In contrast to other currently used methodologies (SEC, FPLC, electrophoresis and SV-AUC), the FFF separation system coupled on-line with UV and MALS detection allows the fractionation of HMW protein aggregates and simultaneous measurement of their MW, r.m.s. radius and molecular conformation (Fig. 4; Tables 2, 3, 5 and 6).

The comparison between the results obtained under native conditions and under mild denaturing conditions is reported in Tables 2 and 3 (native) and Tables 5 and 6 (mild denaturing). The CTR sample and the PQ-treated samples have a common protein range ($MW < 3.5 \times 10^6$ Da); however, only the PQ-treated samples contain protein aggregates with higher MW, and their MW upper limit increases with the PQ concentration. The relative amount (%) of the PQ-induced aggregates increases with the PQ molarity (Table 2). When separated under denaturing conditions, the relative amount of PQ-induced protein aggregates decreases drastically (Table 5). Their size (expressed as hydrodynamic radius and r.m.s. radius) is also reduced under denaturing conditions. The molecular conformation changes with the PQ molarity (Fig. 4b) toward progressively more compact forms. Under denaturing conditions, the protein aggregates in the 10 mM PQ sample assume an even more compact conformation; i.e., they are sphere-like.

The traditional AF4 (a larger version of HF5) long channel could be used for semipreparative purposes (adjusting the flow rates for the separation); however, we believe that the loss of HMW protein aggregates (owing to nonspecific interaction with the membrane wall) would be significant as compared with the HF5 miniaturized device.

The HF5-UV-MALS experimental setup described here allows obtaining the following information: (i) the absolute MW of the PQ-induced aggregates (no standards required), which is a measurement that cannot be accurately attained with any other method; (ii) the r.m.s. radius and molecular conformation of the aggregates, both of which are important factors in determining cellular clearance of the aggregates; (iii) the aggregates' behavior under denaturing conditions, which is important to determine the covalent versus noncovalent nature of the aggregates; and (iv) the relative percentage of cellular aggregates under increasing amount of PQ-induced oxidative stress, which is a measurement that would be extremely useful in monitoring conditions and treatments aimed at decreasing cellular aggregates.

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Figure 1. Schematic of HF5; separation principle and protocol workflow. **(a)** Sample focus step: the sample is concentrated in a narrow band and each sample component reaches its steady state (relaxation), at a specific distance from the channel wall. **(b)** Sample elution step: once the proteins have reached their steady state, the channel flow in longitudinal direction leads the proteins toward the exit of the channel, whereas the radial flow pushes the proteins toward the wall, separating them along the way. **(c)** Workflow for the hydrodynamic-based separation and characterization of protein aggregates from total cell lysates. Highlighted colored boxes correspond to the steps described in the protocol.

Figure 2.

HF5 separation device setup and flow parameters. **(a,b)** View of the HF5 double-length cartridge separation device and flow parameters for the sample focus step **(a)** and detailed view of the focus process inside the separation device **(b)**. **(c)** Sample elution step: flow parameters. **(d)** View of the commercial HF5 cartridge—patent no. DE102010043877B3 (Supern). **(e)** Schematic of the instrumental setup required for the protocol depicting HPLC components (degasser, pump, autosampler and DAD) connected to the FFF separation system (Eclipse DUALTEC module) that hosts the HF5 device and the MALS (DAWN EOS used in this protocol). The PC controls all the instruments through specific software and performs data processing. DAD, diode array detector; FFF, field-flow fractionation; HF5, hollow-fiber flow FFF; MALS, multiangle light-scattering detector; VWD, variable wavelength detector.

Figure 3.

Results: carbonyl content and SDS-PAGE of protein aggregates. **(a)** Carbonyl content of protein lysates collected from cells incubated with increasing concentrations of PQ. **(b)** Silver staining of an SDS-PAGE run with the same protein lysates as in **a** to detect protein aggregates. HMW aggregates and low-MW oxidative cleavage products are observed in the PQ-treated cells.

Figure 4.

Results: Separation and characterization of protein aggregates. **(a)** HF5-UV-MALS of control (CTR) and PQ (0.25, 5 and 10 mM)-treated cell lysates. Overlaid fractograms: thin lines report Rayleigh ratios at 90° (external right y-axis); dash-dotted lines report optical density reading at 280 nm (internal right y-axis); thick lines report calculated molecular weight (MW), displayed in logarithmic scale (left y-axis); x-axes reporting the retention time (top) and corresponding hydrodynamic radius values (bottom). The MW-time of retention (t_R) correlation is represented by red empty circles for the native protein markers and by the red empty triangle for dextran blue (MW is 2×10^6 g/mol). **(b)** Correlation plots: r.m.s. radius values plotted against the corresponding MW values (both in logarithmic scale) of protein aggregates in CTR and PQ (0.25, 5 and 10 mM)-treated cell lysates. Numbers in parentheses correspond to the slope of each plot; each value was assigned to a known conformation type. **(c)** Schematic of fraction collection. **(d)** Silver staining of a native gel of fractionated protein lysates from CTR and 10 mM PQ-treated cells. Separation by MW is observed in progressively higher fractions. Very HMW aggregates are mostly observed in fraction 6 of PQ-treated cells. LS, light scattering.

TABLE 1

Eclipse method setup for HF5^a.

Start time (min)	End time (min)	Duration (min)	Mode	v _x Start (ml/min)	v _x End (ml/min)
0.0	0.5	0.5	Focus		
0.5	5.5	5.0	Focus - Inject		
5.5	75.5	70.0	Elution	0.4	0.1
75.5	80.5	5.0	Elution - Inject	0.0	0.0

Steps 20–23 (system setup), 24–33, and 44 and 45 (analysis of cellular aggregates under native and mild denaturing conditions).

^a Settings: separation device HF5; device properties: fiber radius (mm) 0.4; membrane type PES 10 kDa; flow settings: detector flow (elution flow rate) 0.5 ml/min; focus flow 0.6 ml/min; autofocus position: focusing (%) 20%; v_x represents the cross-flow rate (ml/min).

TABLE 2

Analysis of cellular aggregates under native conditions (Steps 24–33).

Sample	CTR	0.25 mM PQ	5 mM PQ	10 mM PQ
MW range of the CTR and PQ common proteome (g/mol)			1.5×10^4 to 3.3×10^6	
Size range (hydrodynamic radius) of the CTR and PQ common proteome	2–115 nm	2–74 nm	2–61 nm	2–45 nm
MW range of the PQ-induced aggregates (g/mol)		3.3×10^6 to 5.5×10^6	3.3×10^6 to 6.2×10^6	3.3×10^6 to 1.7×10^7
Size range (hydrodynamic radius) of the PQ-induced aggregates		74–115 nm	61–115 nm	45–115 nm
Relative amount ^a of the PQ-induced aggregates		12.5%	17.9%	25.1%

^aRelative to the postfractionation total protein amount.

TABLE 3
Fractionation and analysis of cellular aggregates separated under native conditions (Steps 26–31).

Control samples						
Collected fractions	Figure 4c	1	2	3	4	5
MW range (g/mol)	Figure 4a	4.4×10^4 to 1.5×10^5	1.5×10^5 to 2.8×10^5	2.8×10^5 to 6.5×10^5	6.5×10^5 to 2.2×10^6	2.2×10^6 to 3.0×10^6
Size range (r.m.s. radius; nm)	Figure 4b	NA	NA	NA	20.9–46.6	3.0×10^6 to 3.3×10^6
PQ-treated samples (10 mM)						
Collected fractions		1	2	3	4	5
MW range (g/mol)		1.6×10^4 to 1.6×10^5	1.6×10^5 to 4.8×10^5	4.8×10^5 to 1.2×10^6	1.2×10^6 to 7.2×10^6	7.2×10^6 to 1.0×10^7
Size range (r.m.s. radius; nm)		NA	NA	NA	18.0–52.6	1.0×10^7 to 1.7×10^7

NA, not applicable.

TABLE 4

Troubleshooting table.

Step	Problem	Possible reason	Solution
20	The UV and/or light scattering baseline is not stable	The system conditioning procedure was not complete or there are air bubbles in the system	Continue to flush the system with the carrier solution. Clean the system with cleaning solution, followed by Milli-Q water (1–2 h) and carrier solution (1–2 h), at 0.5–0.6 ml/min
		The system seems to be leaking	Check the system for possible leaks
22	The retention times of the native protein markers have changed substantially (>1 min shift) from the previous run	The fiber is depleted	Change the fiber and perform the fiber conditioning procedure (make up with BSA standard)
		The baseline shows protein peaks	Clean the system with cleaning solution, followed by Milli-Q water (1–2 h) and carrier solution (1–2 h), at 0.5–0.6 ml/min. Perform the BSA standard separation again, and normalize according to the instructions provided in the Astra manual
23	The theoretical and experimental retention times for the native protein markers do not match	ISIS assumes that the proteins have a rigid sphere conformation, whereas under real experimental conditions the protein conformations may be different	Repeat the native protein marker separation and compare it with previous results
27	The MW values are not displayed in EasyGraph or have unrealistic values	The baseline is not correctly set, which is a frequent problem when the protein amounts are very low	Adjust the baseline manually
		The input parameters are incorrect	Check the Astra method (normalization coefficients, baseline setting, detector delay and so on)
		The signals are very noisy and impede the MW calculation	Clean the system as described for Step 22
28	The r.m.s. radius values are not displayed in EasyGraph	The signals are very noisy and impede the r.m.s. radius calculation	Clean the system as described for Step 22/(br)Please note that the lower instrumental limit for the r.m.s. radius calculation is 10 nm
29	The r.m.s. conformation plot is not displayed in EasyGraph	The MW and/or the r.m.s. radius values cannot be calculated	Refer to Steps 25 and 26 in the PROCEDURE
30	The protein amount is not calculated or has an unrealistic value	The Astra input parameters are incorrect	Check the crucial parameter values: the flow rate value and the extinction coefficient value. Please make sure that you select the whole cell lysate peak (considered 100%) and the areas of interest (i.e., the HMW protein fraction eluting from 30 to 40 min) in order to obtain an accurate estimation of the recovered protein amount
		The protein sample was not quantified correctly by the BCA assay	Repeat the quantification and if the issue is not solved, consider adjusting the injection volume
31	The fractions chosen for this protocol are not relevant	The fractions are not tailored to your needs	Astra allows selecting virtual fractions from the Peak menu and calculates parameters such as MW value and r.m.s. radius value for each fraction. Adjust the fractions collection to your specific needs, i.e., in the protein MW range of your interest

TABLE 5

Analysis of cellular aggregates under mild denaturing conditions (Steps 44 and 45).

Sample	CTR	10 mM PQ
MW range of the CTR and PQ common proteome (g/mol)	1.5×10^4 to 3.3×10^6	
Size range (hydrodynamic radius) of the CTR and PQ common proteome	2–30 nm	2–22 nm
MW range of the PQ-induced aggregates (g/mol)	—	3.3×10^6 to 1.7×10^7
Size range (hydrodynamic radius) of the PQ-induced aggregates	—	22–37 nm
Relative amount ^a of the PQ-induced aggregates	—	1.9%
MW range of aggregates induced by denaturing conditions	3.3×10^6 to 1.7×10^7	1.7×10^7 to 4.3×10^7
Size range (hydrodynamic radius) of the aggregates induced by denaturing conditions	30–58 nm	37–58 nm
Relative amount ^a of the aggregates induced by denaturing conditions	8%	0.9%

^aRelative to the postfractionation total protein amount.

TABLE 6
Fractionation and analysis of cellular aggregates separated under mild denaturing conditions (Steps 44–45).

Control samples	1	2	3	4	5	6
Collected fractions						
MW range (g/mol)	7.1×10^3 to 3.4×10^4	3.4×10^4 to 1.4×10^5	1.4×10^5 to 3.1×10^5	3.1×10^5 to 3.1×10^6	3.1×10^6 to 3.3×10^6	
Urea-induced aggregates: MW range (g/mol)					3.3×10^6 to 9.1×10^6	9.1×10^6 to 1.7×10^7
r.m.s. radius range (nm)	NA	NA	NA	NA	16.0–27.6	
Urea-induced aggregates: r.m.s. radius range (nm)					16.2–27.6	27.6–38.9
PQ-treated samples (10 mM)						
Collected fractions						
MW range (g/mol)	6.2×10^3 to 3.9×10^4	3.9×10^4 to 1.2×10^5	1.2×10^5 to 6.8×10^5	6.8×10^5 to 1.0×10^7	1.0×10^7 to 1.7×10^7	
Urea-induced aggregates: MW range (g/mol)					1.7×10^7 to 3.9×10^7	3.9×10^7 to 4.3×10^7
r.m.s. radius range (nm)	NA	NA	NA	7.5–23.6	23.6–26.1	
Urea-induced aggregates: r.m.s. radius range (nm)					26.1–36.8	36.8–49.9

NA, not applicable.