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A fractionation approach applying chelating magnetic nanoparticles to characterize pericardial fluid's proteome

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ABSTRACT

Owing to their close proximity, pericardial fluid (PF)'s proteome may mirror the pathophysiological status of the heart. Despite this diagnosis potential, the knowledge of PF's proteome is scarce. Large amounts of albumin hamper the characterization of the least abundant proteins in PF. Aiming to expand PF's proteome and to validate the technique for future applications, we have fractionated and characterized the PF, using N-(trimethoxysilylpropyl)ethylenediamine triacetic acid (EDTA)-functionalized magnetic nanoparticles (NPs@EDTA) followed by a GeLC-MS/MS approach. Similarly to an albumin-depletion kit, NPs@EDTA-based fractionation was efficient in removing albumin. Both methods displayed comparable inter-individual variability, but NPs@EDTA outperformed the former with regard to the protein dynamic range as well as to the monitoring of biological processes. Overall, 565 proteins were identified, of which 297 (>50%) have never been assigned to PF. Moreover, owing to this method's good proteome reproducibility, affordability, rapid automation and high binding ability of NP@EDTA, it bears a great potential towards future clinical application.

1. Introduction

Pericardial fluid (PF) is a plasma ultrafiltrate enclosed in the pericardial sac, providing lubrication during heartbeat [1]. Albeit not commonly used in the clinical routine, PF in close proximity to the heart, likely holds relevant molecular information, reflecting its pathophysiological status. Even though, the molecular knowledge of PF is rather scarce. The need for an invasive collection and the ethical restraints of collecting PF from healthy subjects have been probably discouraging its study. However, PF can be easily and safely collected, for instance, from patients undergoing open heart surgery [2]. Furthermore, once it is enriched with many bioactive substances, such as cytokines, growth factors or cardiac hormones, governing heart function by autocrine or paracrine activity, PF provides a direct window to the heart. Therefore, the molecular analysis of PF may be an important tool to address the pathophysiological mechanisms underlying cardiac diseases and, simultaneously, to pinpoint potential therapeutic targets [2,3].

Proteomics provides a reliable approach to study PF because the profiling (and quantification) of biofluid proteins allows us to scrutinize directly the phenotype of diseases. Nonetheless, the proteomic characterization of PF can be challenging. First, the albumin content can be even higher than in plasma (71% versus 62%) [3], thus the probability of albumin masking other less abundant

Abbreviations: BCA, bicinchoninic acid; CAD, coronary artery disease; EDTA, N-(trimethoxysilylpropyl)ethylenediamine triacetic acid; EDTA-TMS, N-(trimethoxysilylpropyl)ethylenediamine triacetate trisodium salt; FA, formic acid; FDR, false discovery rate; FTIR, Fourier Transform Infrared; GO, gene ontology; ID, internal diameter; IDA, information-dependent acquisition; LC, liquid chromatography; MARS, multiple affinity removal system; MES, 2-(N-Morpholino)ethanesulfonic acid; MS, mass spectrometry; PF, pericardial fluid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TEM, transmission electron microscopy; TOF, time-of-flight.

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proteins needs to be accounted for. Second, PF is expected to bear a high dynamic range of protein concentrations because it is generated by plasma ultrafiltration. The plasma itself displays a range higher than 10^{10}-fold [4]. Third, data integration will be difficult since we have available only one study published so far, reporting the first in-depth proteomic characterization of PF [5]. In that study the first 1000 PF proteins were identified by removing the 14 most abundant proteins (using Agilent's Multiple Affinity Removal System, MARS) and by following a SDS-PAGE-LC-MS/MS approach. Still, the removal of so many proteins, e.g. albumin, IgM or apolipoprotein AI, was made at the expense of other less abundant small proteins/peptides, which bind to the column-captured proteins. Besides, such method is highly expensive due to the antibody dependency, which readily hampers large-scale use in clinical research.

We have previously used magnetic nanoparticles functionalized with N-(trimethoxysilylpropyl)ethylenediamine triacetic acid (EDTA) (NPs@EDTA) to obtain metal-dependent proteins-enriched salivary fraction from chronic periodontitis subjects to perform gelatin zymography. In such assay, NPs@EDTA proved to be useful for metalloproteases enrichment as evidenced by enhanced metalloproteases-driven gelatinolytic activity [6]. The ability to enrich metalloproteins or metal-dependent proteins is explained by the EDTA's chelating properties, a pentadentate ligand anchored in the surface of magnetic nanoparticles. Additionally, these nanoparticles display tremendous potential for clinical application owing to their affordability, high surface area-to-volume ratio and, thus, sensibility, in addition to the ability to provide a fast separation upon application of an external magnetic field [7].

We hypothesized that processing PF with NPs@EDTA would deepen PF's proteome knowledge, by enriching low abundant proteins. Therefore, we aimed to validate the use of NPs@EDTA to fractionate and characterize PF proteome and also to expand the proteome catalogued to date. In order to do that, we have compared the application of these lab-made nanoparticles with a commercial albumin/IgG-depletion kit, with regard to the SDS-PAGE profiles as well as to the profile of protein fractions and to the trackable biological processes.

2. Materials and methods

2.1. Sample collection, processing and protein quantification

PF was collected from two obese, hypertensive and diabetic female patients during valve surgery (samples were named PF1 and PF2). A third sample was collected from an obese, hypertensive non-diabetic male patient during valve surgery (named PF3). Approximately 15 mL of fluid was collected from each patient immediately after sternotomy and pericardiotomy. Following collection, PF was centrifuged at 5000 rpm, 4 °C for 15 min to remove cellular components and the supernatant was stored at -80 °C until further processing. Protein quantification was performed with the BCA protein assay kit (Pierce®, Thermo Scientific). The study was approved by the Institutional Ethical Committee, and all patients provided written informed consent.

2.2. Depletion of albumin and immunoglobulin G from pericardial fluid

PF from subjects PF1 and PF2 was processed using an albumin/IgG-depletion kit (Pierce™ Top 2 Abundant Protein Depletion Spin Columns, Thermo Scientific), according to the manufacturer's protocol. The volume corresponding to 600 μg of proteins was used since the spin columns can process up to 10 μL of serum/plasma (~600 μg of protein). An additional elution step was performed in order to collect the albumin/IgG-rich fraction. To collect these fractions, samples were incubated with 300 μL of 0.1 M glycine solution pH 2.4, for 30 min, in an end-over-end mixer, and centrifuged for 2 min at 1000 × *g*. Neutralization of samples was achieved with 0.5 M Tris solution pH 8.5.

2.3. Synthesis and characterization of NPs@EDTA

A detailed description of NPs@EDTA (patent register PT107608) preparation has been described elsewhere [7] but, essentially, comprised: i) the synthesis of the magnetic core made of Fe_3O_4 through oxidative hydrolysis of an Iron (II) salt in alkaline medium; ii) surface coating with a shell of amorphous silica (SiO_2) in alkaline environment using tetraethyl *ortho*-silicate as a precursor; and iii) surface chemical functionalization with N-(trimethoxysilylpropyl) ethylenediamine triacetate trisodium salt (EDTA-TMS), as illustrated in Figure S1.

Morphology and size of NPs@EDTA particles were obtained through transmission electron microscopy (TEM) using a Hitachi H-9000 microscope operated at 300 kV. Sample for TEM analysis was prepared by evaporating dilute suspensions of the nanoparticles on a copper grid coated with an amorphous carbon film. The Fourier Transform Infrared (FTIR) spectrum was collected using a spectrometer Mattson 7000 with 256 scans and 4 cm^{-1} resolution, using a horizontal attenuated total reflectance cell. The surface charge of the NPs was assessed by zeta potential measurements, using a Zetasizer Nanoseries equipment from Malvern Instruments.

2.4. NPs@EDTA assay for fractionation of pericardial fluid proteins

After NPs@EDTA synthesis, a suspension of nanoparticles was prepared in ultrapure water to a known concentration. One mg of NPs@EDTA was used to each sample. Before incubation of samples, magnetic beads were washed with binding buffer [0.01M 2-(N-Morpholino)ethanesulfonic acid (MES), 0.01M NaCl, pH 6.5–8.5]. Then, two independent assays were performed, using samples PF1 and PF2. In the first, 0.6 mg (the same amount processed with the commercial kit) and 3 mg of PF protein were incubated with the beads and subjected to mechanical agitation for 1 h at room temperature. In the second, to estimate NPs@EDTA saturation, 0.3 mg, 1.5 mg, 3 mg and 4.5 mg of PF proteins were incubated with the beads and subjected to the same agitation conditions. After sample incubation, supernatants were collected and the beads were washed 3 times by adding 500 μL of MES buffer and performing agitation cycles for 3-5 min. Elution of beads-bound proteins was carried out with 20 μL of Laemmli loading buffer by promoting further mechanical agitation for 10 min.

2.5. Protein separation by SDS-PAGE

Aiming to compare the protein profiles of the fractions collected with the commercial kit and with the NPs@EDTA, protein fractions were separated by SDS-PAGE, following Laemmli procedure [8]. The volume corresponding to 30 μg of protein from total unprocessed sample and from the protein fractions collected by the commercial kit was directly loaded onto gel. Ten μL of each fraction collected in the first NPs@EDTA assay was also loaded onto gel. This volume was chosen because, when starting with 600 μg of protein, it corresponds to the same protein amount (30 μg) as in the case of the kit. Finally, in the second (saturation) assay the whole adsorbed protein fractions were loaded onto gel. Proteins were separated under reducing and denaturing conditions in 12.5% gels, applying a 200 V voltage. Gels were then incubated in fixation solution (methanol: acetic acid 40:10 v/v) for 45 min, stained with Colloidal Coomassie Blue G250 and destained with 20% methanol until an optimal