An Affinity-Based Workflow for Clusterin Purification and Interactome Characterisation in Human Plasma

AUTHOR NAMES

Amalia Kontochristou, Nika Šimičić, Anouk M. Rijs, and Melissa Baerenfaenger*

AUTHOR ADDRESS

Amalia Kontochristou − Division of Bioanalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, Amsterdam Institute of Molecular and Life Sciences, Vrije Universiteit Amsterdam, 1081 HV Amsterdam, The Netherlands; Centre for Analytical Sciences Amsterdam (CASA), The Netherlands; orcid.org/0009-0006-5988-0024

Nika Šimičić − Division of Bioanalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, Amsterdam Institute of Molecular and Life Sciences, Vrije Universiteit Amsterdam, 1081 HV Amsterdam, The Netherlands; Centre for Analytical Sciences Amsterdam (CASA), The Netherlands; Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden 2333, ZA, The Netherlands; orcid.org/0009-0000-4381-6219

Anouk M. Rijs − Division of Bioanalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, Amsterdam Institute of Molecular and Life Sciences, Vrije Universiteit Amsterdam, 1081 HV Amsterdam, The Netherlands; Centre for Analytical Sciences Amsterdam (CASA), The Netherlands; orcid.org/0000-0002-7446-9907

Melissa Baerenfaenger − Division of Bioanalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, Amsterdam Institute of Molecular and Life Sciences, Vrije Universiteit Amsterdam, 1081 HV Amsterdam, The Netherlands; Centre for Analytical Sciences Amsterdam (CASA), The Netherlands; orcid.org/0000-0002-2855-924

KEYWORDS

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ABSTRACT Clusterin is an extracellular chaperone protein involved in maintaining proteostasis by binding to unfolded or misfolded proteins to prevent their aggregation. Its chaperone activity plays a significant role in human health, as seen in Alzheimer's disease, where clusterin co-localises with amyloid-β plaques to facilitate their clearance. However, its strong tendency to "cluster" with a wide range of client proteins and its low abundance in biological matrices complicate its characterisation. To enable comprehensive analysis of clusterin in human plasma, we established two affinity-based purification workflows, one for targeted clusterin purification and a second for interactome analysis, combining affinity enrichment with bottom-up proteomics. Systematic optimisation of the purification workflow revealed that disrupting ionic and hydrophobic protein-protein interactions was essential to improve clusterin enrichment while minimising co-purification. In contrast, preserving native protein-protein interactions enabled clusterin interactome analysis using affinity purification-mass spectrometry (AP-MS). Bottom-up proteomics profiling identified proteins within the clusterin interactome involved in immune response, hemostasis, and high-density lipoprotein (HDL)-related pathways. Together, these complementary workflows enable targeted clusterin

purification and systematic characterisation of its interactome, offering new insights into clusterin's biological roles in human plasma.

Introduction

Clusterin (CLUS) is a glycosylated protein with a molecular weight of 60-85 kDa, expressed both intracellularly and extracellularly across almost all tissues and biofluids, including plasma, cerebrospinal fluid, saliva, and urine[1,2]. As a molecular chaperone, clusterin binds to hydrophobic regions exposed on misfolded or stress-damaged proteins, such as amyloid-β, α-synuclein, and Tau[3–6], and either stabilises them or promotes their degradation[7,8]. By shielding these hydrophobic segments, clusterin suppresses protein aggregation and limits the pathological accumulation of amyloidogenic proteins in cells and biofluids, making it an attractive target for studying mechanisms of neurodegeneration in Alzheimer's and Parkinson's disease[9,10]. Beyond that, clusterin participates in various other biological processes, including modulation of inflammation via negative regulation of the complement system[11], association with fibrin clots during blood coagulation[12], transport of proteins across the blood-brain barrier[13], and incorporation into high-density lipoprotein particles in plasma[2,14]. Due to its broad tissue distribution, diverse structure, and functional versatility, clusterin has been the focus of many studies[15–18]. Yet, its purification from biological samples remains challenging.

Various approaches have been employed to purify clusterin from human plasma, consistently highlighting the challenge posed by both the intrinsic complexity of the biofluid[19] and clusterin's known ability to form complexes with a wide range of proteins[20–22], including immunoglobulins, apolipoproteins, and complement components[23,24], that further complicates its isolation. Human plasma contains thousands of proteins spanning a wide dynamic range, with a small number of highly abundant species, such as albumin and immunoglobulin G,

accounting for more than 90% of the total protein content[19,25]. In contrast, clusterin is present in plasma at substantially lower levels, such that without prior enrichment, detection and characterisation are severely hindered by dominant plasma proteins[26]. To address these challenges, many purification protocols rely on immunoaffinity enrichment[27,28] using monoclonal or polyclonal antibodies, which provide high binding specificity for clusterin[29,30], often in combination with complementary chromatographic techniques, such as size-exclusion chromatography[30], ion-exchange chromatography[31], and hydrophobic interaction chromatography[32]. These techniques preserve the native structure of clusterin and consequently maintain its chaperone activity, which is crucial for functional studies or interactome analysis. However, the retained chaperone activity complicates purification, as clusterin continues to bind its client proteins, resulting in extensive co-purification[33]. This obstacle was also observed by Gordon *et al.*[34], who co-purified clusterin with several plasma proteins despite optimised separation, highlighting the difficulty of isolating clusterin in its native form due to its chaperone function.

To overcome these challenges, we established a workflow that integrates immunoaffinity enrichment and bottom-up proteomics to optimise clusterin purification and enable characterisation of its interaction network in human plasma. Clusterin enrichment was achieved using a commercially available clusterin-specific affinity resin containing recombinant single-domain antibody fragments comprising solely the variable-heavy domain, and it was implemented in two modes. Under protein-protein interaction-disruptive conditions that minimise ionic and hydrophobic interactions, the workflow enabled selective purification of clusterin, while minimising co-purification of other proteins. In contrast, when operated under milder, non-disruptive conditions, the same workflow was used for interactome profiling by affinity purification-mass spectrometry. AP-MS is a well-established technique for mapping protein-protein interactions, in which a bait protein, in this case clusterin, is isolated along with

its binding partners[35], thereby enabling the detection of stimulus- and condition-dependent protein associations[36,37]. These workflows therefore provide complementary insight into both the selectively enriched protein and its dynamic interaction network in plasma, thereby informing clusterin's functional role in health and disease.

Materials and Methods

Purification of human plasma CLUS

Pooled human plasma (S4180-100) was sourced from Biowest SAS (Nuaillé, France). For the enrichment of clusterin from 50 µL of human pooled plasma, 100 µL of POROS™ CaptureSelect™ ClusterinClear Affinity Resin (10 mL), kindly provided by Thermo Fisher Scientific (Carlsbad, CA, USA), was used. The clusterin affinity resin was transferred into a Pierce™ Micro-Spin Column supplied by Thermo Fisher Scientific (Rockford, USA). Before applying the plasma sample, the resin was first washed with Milli-Q water and equilibrated with Dulbecco's Phosphate Buffered Saline (DPBS, pH 7.4) supplied by Thermo Fisher Scientific (Paisley, UK). Milli-Q water was produced by a Millipore purification system (Millipore, Amsterdam, The Netherlands). Human plasma was then diluted 1:1 with DPBS and loaded onto the resin. To remove the unbound proteins, the resin was washed multiple times with DPBS buffer and Milli-Q water. Additional wash steps with sodium chloride (NaCl) and Tween 20, supplied by Sigma-Aldrich (St. Louis, USA), were implemented to minimise protein-protein interaction. Following the washing steps, clusterin was eluted from the affinity material with 100 µL of glycine buffer (pH 2.3), and the eluate was neutralised with 40 µL of Tris(hydroxymethyl)aminomethane (Tris) buffer (pH 9.0), both supplied by Sigma-Aldrich (St. Louis, USA). The sample was then dried under vacuum and stored at −20 °C before bottom-up proteomics analysis. The different purification protocols used are summarised in the Supporting Information (Table S1).

Bottom-up proteomics

The clusterin-enriched sample was subjected to tryptic digestion. For this, the sample was redissolved in 5 µL of 5 M guanidine hydrochloride and 10 µL of 50 mM DL-dithiothreitol, followed by an incubation at 37 °C with shaking at 650 rpm for 20 minutes. Subsequently, 10 µL of 50 mM iodoacetamide was added, followed by a 60-minute incubation in the dark. Then, 60 µL of 50 mM ammonium bicarbonate and 1 µL of Trypsin/Lys-C (1 mg/mL), supplied by Promega Benelux B.V. (Leiden, The Netherlands), were added, and the sample was incubated overnight at 37 °C with shaking at 650 rpm. Trypsin was inactivated by incubation at 90 °C for five minutes. After tryptic digestion, the sample was stored at −20 °C prior to analysis.

Liquid chromatography-tandem mass spectrometry analysis was performed using an Agilent 1290 Infinity II system (Agilent Technologies Netherlands B.V., Middelburg, The Netherlands) coupled to a trapped ion mobility quadrupole time-of-flight (timsTOF Pro 2) mass spectrometer equipped with an electrospray ionisation (ESI) source operating in positive ion mode (Bruker Daltonics, Bremen, Germany). LC separation was performed at 55 °C with a flow rate of 80 µL/min using solvent A (0.1% formic acid (FA) in water) and solvent B (0.1% FA in acetonitrile). The column used was an Acclaim PepMap 100 C18 reversed-phase column (1 mm ID × 150 mm length, 3 µm particles, 100 Å pore size) supplied by Thermo Fisher Scientific (Ermelo, The Netherlands). The gradient was set as follows: 0 min, 3% B; 2 min, 3% B; 37.5 min, 40% B; 38 min, 90% B; 43.5 min, 90% B; and 60 min, 2% B. Prior to sample injection, mass and mobility calibration were performed using Agilent Technologies ESI-L tuning mix ions. For MS analysis, the DDA PASEF-standard_1.1sec_cycletime.m Bruker application method was used, with adapted ESI source settings [38]. More information on the MS settings is provided in the Supporting Information (Table S2).

Data Processing

For data analysis, FragPipe v23.0, MSFragger version 4.3, and Philosopher v5.1.1 were used[39]. Label-free quantification with match between runs (LFQ-MBR) was performed using the IonQuant workflow (version 1.11.11). The UniProt ID UP000005640 database downloaded on 9 December 2023 was used. Precursor mass tolerance was set at ±20 ppm, and trypsin and Lys-C were selected as the enzymes for proteolytic digestion. Carbamidomethylation of cysteine was set as a fixed modification, while methionine oxidation, pyroglutamic acid formation, or loss of ammonia at the peptide N-terminus, and N-terminal acetylation were specified as variable modifications. For MS1 quantification, the MaxLFQ minimum ions was set to 3, the match-between-runs ion false discovery rate (MBR ion FDR) was set to 0.01, and peptide-protein uniqueness was restricted to unique only.

The generated data was processed and visualised using in-house developed Python scripts, which are available upon request. To visualise the distribution of protein abundances across the samples, MaxLFQ Intensity values were used. For each protein, the mean MaxLFQ Intensity was calculated across replicates, and proteins with non-positive mean values were excluded. Then, the mean intensities were $\log_{10}$-transformed, sorted in descending order, and plotted as abundance rank versus $\log_{10}$(MaxLFQ Intensity) using matplotlib.pyplot library version 3.10.0.

For bar plot generation, the relative abundance of clusterin was calculated for each replicate as the percentage of its MaxLFQ Intensity relative to the total MaxLFQ Intensity across all proteins within the sample. Relative clusterin abundances were summarised across experimental conditions and visualised as bar plots using seaborn version 0.13.2 and matplotlib.pyplot libraries.

For the volcano plots, the MaxLFQ Intensity of proteins was $\log_2$-transformed, and median-centred normalisation was applied per condition to correct for systematic variation. Missing

values were imputed per condition using a normal distribution with a downshift of 1.8 standard deviations and a width of 0.3 times the standard deviation of the observed values, following a Perseus-style approach. Welch's t-test was used to compare conditions, and $\log_2$ fold changes (FC) were calculated from the mean normalised intensities. Volcano plots were generated using the seaborn and matplotlib libraries, with $\log_2$FC on the x-axis and $-\log_{10}$(p-value) on the y-axis. Proteins with $|\log_2 FC| \geq 1$ and $p < 0.05$ were considered significantly enriched.

To define the interactome of clusterin, proteins significantly enriched after clusterin purification relative to non-enriched human plasma, as identified by volcano plot analysis, were selected and analysed using the STRING database version 12.0. Details of the STRING parameters and settings used for network generation are provided in the Supporting Information (Table S3).

Results and Discussion

Clusterin affinity purification

Clusterin was purified from human plasma using a clusterin affinity resin containing immobilised antibodies in a solid-phase extraction (SPE) setup. The purification process can be summarised into three main steps, as shown in Figure 1. First, the sample was loaded onto the affinity resin, allowing clusterin to bind to the immobilised antibody. This was followed by a series of washing steps to remove unbound components. Finally, clusterin was eluted from the affinity resin by lowering the pH, thereby disrupting the antibody–antigen binding.

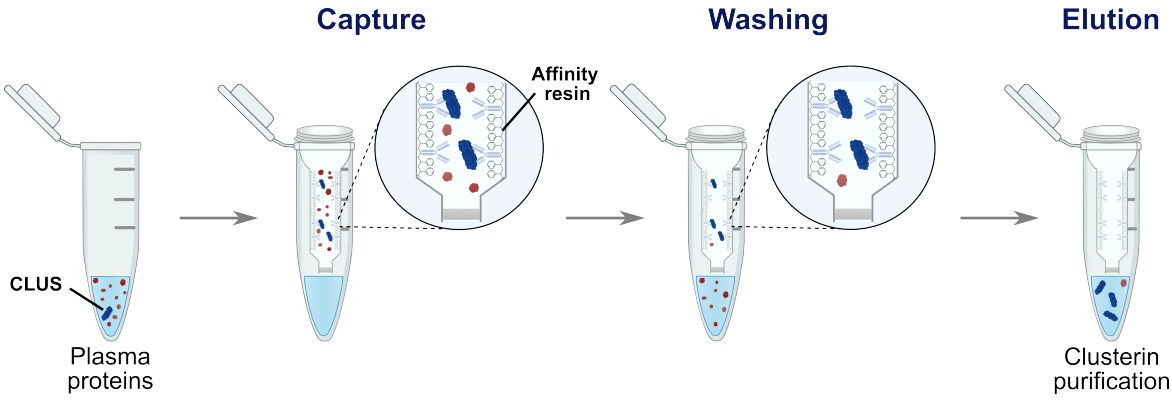

Figure 1: Clusterin purification workflow using POROS™ CaptureSelect™ ClusterinClear affinity resin. The purification was performed in a spin column and involved three sequential steps. Capture: Human plasma was loaded onto the affinity resin, where clusterin (CLUS) bound selectively to the immobilised antibody. Washing: Unbound plasma proteins (in red) were removed. Elution: The interaction between clusterin and the antibody was disrupted, resulting in clusterin elution.

The initial clusterin purification protocol evaluated in this study, referred to as Protocol 1, was established according to the supplier's instructions for the clusterin affinity resin adapted for an SPE approach. Similar conditions have also been applied in previous protein purification studies, both for the affinity purification of clusterin complexes[24], and for the purification of other human proteins, such as immunoglobulins[40,41]. According to Protocol 1, DPBS buffer at pH 7.0–7.5 was used as the equilibration buffer to adjust the pH of both the resin and the plasma sample within the recommended range (pH 6–8) prior to sample loading. After sample loading, the resin was washed with DPBS buffer to remove unbound plasma components while retaining clusterin bound to the affinity resin. Finally, clusterin was eluted using a low-pH solution, specifically 0.1 M glycine at pH 2.3, thereby disrupting the clusterin-antibody interaction and releasing the target protein. The purified samples were subsequently subjected to proteolytic digestion and analysed by bottom-up proteomics using label-free quantification.

To assess the effectiveness of the purification, the relative abundance of proteins in the purified sample was compared with that in non-enriched human plasma. For this purpose, the $\log_{10}$(MaxLFQ Intensity) of all identified proteins were plotted against their abundance ranks to illustrate the dynamic range of protein abundances. As shown in Figure 2, Protocol 1 resulted in significant purification of clusterin compared to the non-enriched plasma, as indicated by the decrease in clusterin's abundance rank and by the reduced total number of proteins detected after purification. However, despite the use of a clusterin-specific affinity resin, a significant number of proteins co-purified with clusterin (Figure 2B). Proteins such as albumin (ALBU), immunoglobulin (IGHG1), apolipoprotein (APOA1), and fibrinogen (FIBB) remained more abundant than clusterin even after purification, potentially due to their inherent high abundance in human plasma, suggesting that further purification was required.

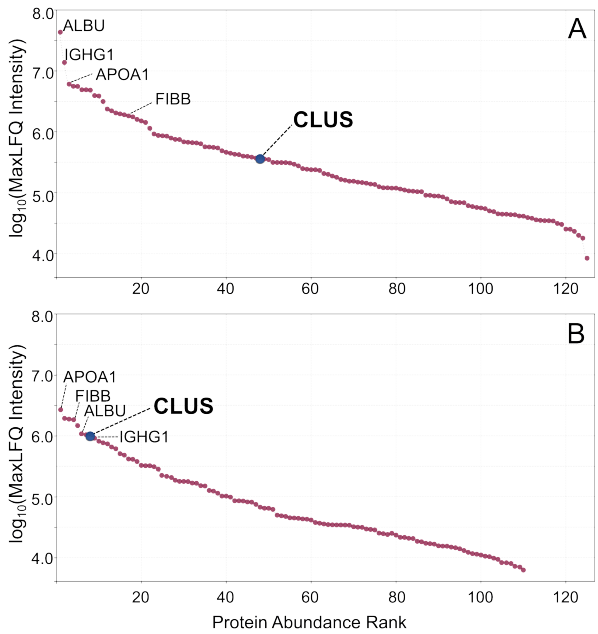

Figure 2: Protein abundance plots of (A) non-enriched and (B) clusterin-enriched human plasma following Protocol 1 for clusterin purification. Proteins were ranked by abundance on the x-axis, while the y-axis displays the average $\log_{10}$(MaxLFQ Intensity) for each protein.

Each dot represents an individual protein. Selected highly abundant proteins (ALBU, IGHG1, APOA1, and FIBB) were highlighted with clusterin (CLUS) shown in blue.

Workflow optimisation

To improve clusterin purification, all steps of the immunoaffinity purification workflow, including capture, washing, and elution conditions, were systematically optimised. To ensure reproducibility and maximise interactions between clusterin and the immobilised antibodies, the affinity resin was kept fully suspended and thoroughly mixed with the sample and buffers at each step of the workflow. In accordance with the supplier's recommendations and to approximate physiological pH, all buffers used for the capture and washing steps were adjusted to pH 7.4, while the elution buffer was maintained at pH 2.3. The pH of the sample was similarly adjusted to approximately 7.4 by diluting 1:1 with DPBS buffer before sample loading. Different incubation times following sample loading onto the affinity resin were also tested to evaluate whether prolonged clusterin-resin contact enhanced clusterin enrichment. However, no significant effect was observed across the different tested incubation times (see Figure S1).

Following these adjustments, various washing buffer compositions were evaluated to further optimise clusterin purification by minimising non-specific ionic and hydrophobic protein-protein interactions that contributed to extensive co-purification of plasma proteins. To achieve that, the concentrations of NaCl and Tween 20 in the DPBS washing buffer were systematically varied. NaCl was first evaluated for its ability to suppress ionic interactions by shielding protein surface charges. NaCl was selected because it is a component of DPBS, and since both Na^+ and Cl^- ions lie at the centre of the Hofmeister Series. They are therefore expected to exert only a minor effect on protein structure and stability[42,43]. Subsequently, Tween 20 was assessed as an additional washing buffer additive to disrupt hydrophobic interactions by coating exposed

hydrophobic regions of proteins, thereby preventing aggregation and non-specific protein-protein interactions involving clusterin[44]. Tween 20 is a non-ionic detergent reported to exert a "renaturating effect" on proteins, referring to its capacity to maintain proteins in solution without inducing denaturation[45]. When evaluated individually, Tween 20 resulted in a greater improvement in clusterin purification than NaCl alone. However, the combined use of both yielded the highest degree of purification (Figure S2).

Multiple combinations of NaCl and Tween 20 concentrations were tested by varying one component at a time while keeping the other constant, to identify conditions that maximised clusterin enrichment. Figure 3 shows the effect of different concentrations of NaCl and Tween 20 on clusterin purification. The detailed purification workflows used are provided in the supporting information (Table S1). As shown in Figure 3A, the relative abundance of clusterin increased with increasing NaCl concentration, reaching a maximum at 0.75 M NaCl. Further increases to 1.0 M and 1.5 M NaCl did not improve purification efficiency but instead led to a decline in clusterin relative abundance. This result likely reflects a salting-in effect, in which a moderate increase in ionic strength shields protein surface charges, reducing protein-protein ionic interactions. Although at higher salt concentrations, competition between salt ions and proteins for water molecules can reduce protein solubility by limiting hydration shell formation around proteins, thereby promoting protein–protein interactions and precipitation[46]. Therefore, higher NaCl concentrations were not tested to avoid potential protein precipitation effects and to prevent interference with mass spectrometry analysis.

In the case of Tween 20 (Figure 3B), progressively increasing its concentration while keeping the NaCl concentration constant enhanced clusterin purification, likely due to the disruption of hydrophobic protein-protein interactions at higher detergent levels[47]. The most substantial improvements were observed between Tween 20 at 0.5% and 2%. Specifically, increasing the concentration of Tween 20 from 0.5% to 1% resulted in a 1.6-fold increase in

clusterin relative abundance, followed by an additional 1.1-fold increase when the Tween 20 concentration was further increased to 2%. Further raising the Tween 20 concentration to 5% yielded only a minor additional improvement in clusterin purification. Considering the limited gain and aiming to avoid protein denaturation[48], 2% Tween 20 was selected as the optimal concentration.

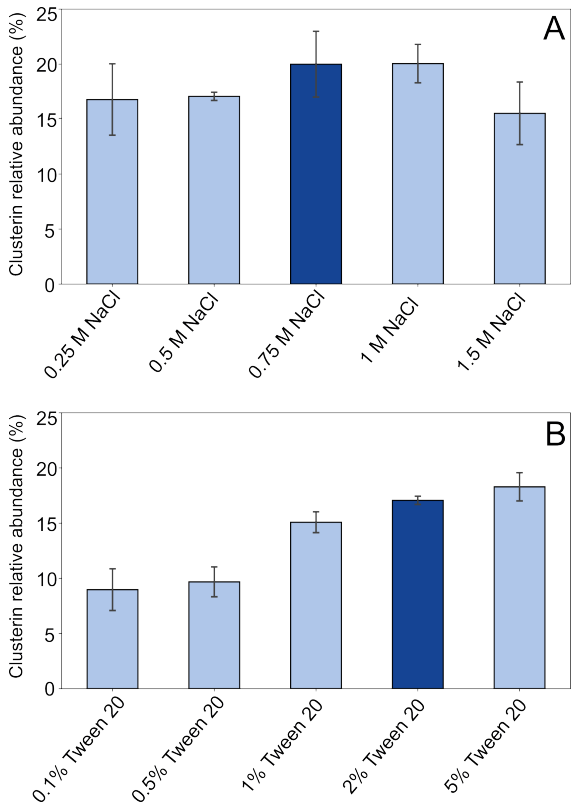

Figure 3: Optimisation of washing buffer composition for clusterin purification. Bar plots show the effect of varying concentrations of (A) NaCl and (B) Tween 20 in the washing buffer on the relative abundance of clusterin. The x-axes indicate the concentrations of NaCl and Tween 20 tested. The y-axes represent the ratio of the $\log_{10}$(MaxLFQ Intensity) of clusterin to the $\log_{10}$(MaxLFQ Intensity) of all proteins in the sample, reflecting relative clusterin abundance. During Tween 20 optimisation, the NaCl concentration was held constant at 0.5 M, while during NaCl optimisation, the Tween 20 concentration was maintained at 2%. Error bars represent standard deviation across three replicates.

Based on the results, the purification protocol was refined as Protocol 2, which incorporated a total of eight washing steps. After the sample was loaded onto the affinity resin, the first two washes were performed with DPBS containing 0.75 M NaCl and 2% Tween 20, followed by two washes with DPBS containing only 0.75 M NaCl to further reduce protein co-purification and remove residual Tween 20. Subsequently, four consecutive washes with Milli-Q water were implemented to remove any remaining Tween 20 and NaCl, ensuring MS compatibility and facilitating the transition to low-pH elution conditions, as Milli-Q water lacks buffering capacity.

After optimising the washing step, the elution buffer composition was also modified to further enhance clusterin purification. To disrupt the interaction between clusterin and antibody, a glycine buffer at pH 2.3 was used, and 0.5 M NaCl was added to improve elution efficiency. The addition of NaCl to the elution buffer resulted in a significant decrease in the co-purification of plasma proteins, as shown in Figure S2D, potentially due to the salting-out effect under acidic conditions. At low pH, most plasma proteins carry a net positive charge and repel one another[49], thereby maintaining their solubility. The addition of salt shields these surface charges, reducing electrostatic repulsion and promoting protein–protein interactions. This increases the likelihood of aggregation or precipitation, thereby lowering protein solubility[46] which could explain the reduction of co-eluted proteins with clusterin.

All the optimisations described above were incorporated into the final purification workflow, referred to as Protocol 3. Compared with the initial protocol (Protocol 1), clear differences were observed in clusterin enrichment and in the extent of protein co-purification, as illustrated in Figure 4. Using Protocol 1, clusterin was enriched, but extensive co-purification of other plasma proteins was also evident (Figure 4A). In contrast, implementation of the optimised workflow (Protocol 3) resulted in a drastic reduction in co-purified proteins, indicating substantially improved purification (Figure 4B). Nevertheless, even under the optimised

condition of Protocol 3, a small number of proteins, including serum paraoxonase/arylesterase 1 (PON1), cysteine-rich secretory protein 3 (CRIS3), several immunoglobulin variable regions (HV118, HV461, KV315, KVD20), peroxiredoxin-5, mitochondrial (PRDX5), complement C1r subcomponent-like protein (C1RL), and serum amyloid A-1 protein (SAA1), co-purified with clusterin. However, their enrichment was substantially lower than that of clusterin, as reflected by smaller fold changes and reduced statistical significance, indicating a weaker and less specific association with the affinity resin.

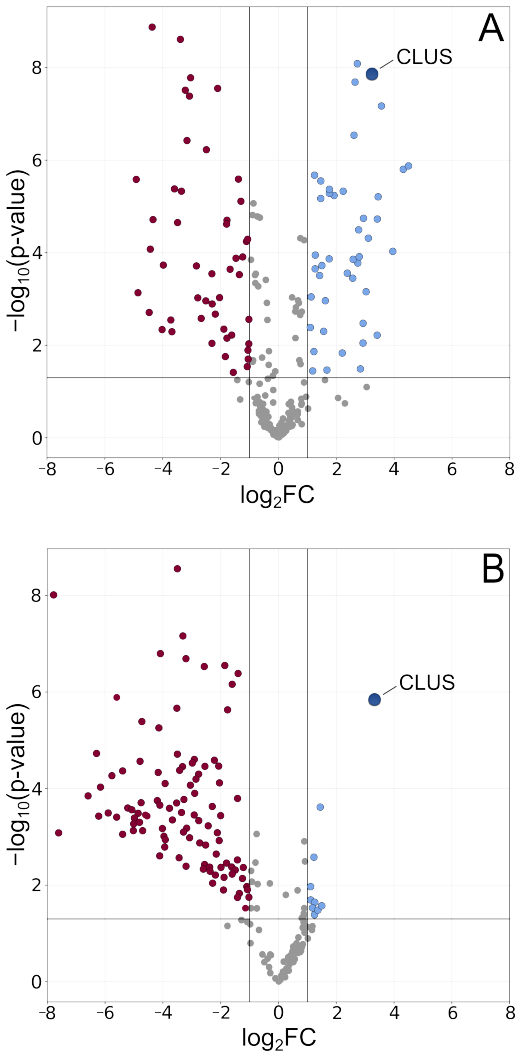

Figure 4: Comparison of clusterin enrichment from human plasma using (A) Protocol 1 and (B) Protocol 3. Volcano plots show $\log_2$FC (x-axis) versus $-\log_{10}$(p-value) (y-axis). Proteins

significantly enriched by clusterin affinity purification are shown in blue, while proteins detected at higher abundance in non-enriched human plasma are shown in red.

Clusterin Interactome

In addition to purification, the affinity resin was used to study the interactome of clusterin. Under non-disruptive conditions, AP-MS enabled the co-enrichment of clusterin with its associated proteins, providing insights into clusterin's interaction network in plasma. For interactome analysis, Protocol 1 was used, as it employed mild purification conditions that preserve protein-protein interactions. Following enrichment, clusterin-associated proteins were identified using bottom-up proteomics and subjected to STRING analysis, which facilitated the visualisation of the predicted and experimentally validated interactions between clusterin and other plasma proteins, based on both physical and functional associations[50].

According to the STRING network presented in Figure 5, clusterin was part of an extensive protein-protein interaction network. Clusterin was directly and indirectly associated with numerous proteins, which could be grouped into three categories based on their biological roles, including proteins associated with the hemostasis pathway, immune modulation, and high-density lipoprotein particles.

In detail, the interactome of clusterin comprised numerous proteins associated with HDL particles, complexes composed mainly of phospholipids, cholesterol, and apolipoproteins, whose primary function is to transport lipids between cells[51]. One of the apolipoproteins present in HDL is clusterin (also known as apolipoprotein J), which mainly facilitates the folding of unfolded proteins[52]. Other proteins associated with HDL that were present in the clusterin interactome include APOA1, APOB, APOE, APOC4, TTHY, SAA4, PON, GPX3, HPTR, CETP, and PLTP[24,52–59].

In addition to HDL particles, several proteins involved in the hemostasis pathway were also identified within the clusterin interactome. This included proteins such as COMP, THBS, FIBB, FIBA, FINC, VTNC, APOE, FBLN1, THRB, and VWF[60–62]. Additionally, coagulation factors such as F13A, F13B, FA9, and PROC, along with its cofactor PROS1[60,61,63]. The abundance of hemostasis-related proteins in the interactome of clusterin may be attributed to the role of extracellular chaperones in fibrin clots. While not directly participating in the coagulation cascade, chaperones, like clusterin, are thought to facilitate the correct folding of unfolded or misfolded proteins, thereby delaying or inhibiting clot formation and exerting a potential anticoagulant effect[64]. Moreover, studies in endothelial cells also suggest a connection between clusterin and the regulation of hemostatic mediators[65,66].

The third group of proteins identified in the interactome included proteins involved in the immune response pathways. This group included proteins such as SAA4 and LBP, which are associated with the innate immune response as acute-phase proteins[67]. Other proteins in this group comprised those participating in the complement cascade, such as CO4A, CO4B, C4BPA, C4BPB, C1S, and CO5[68]. The complement system is a vital component in the defence mechanism of the human body, responsible for tagging pathogens for destruction, activating immune cells, and directly eliminating pathogens through the membrane attack complex (MAC) mechanism[69,70]. Clusterin has been reported to inhibit MAC formation, thereby protecting host cells from complement-mediated damage and preventing the lysis of healthy, non-target cells, together with another protein present in the interactome, VTNC[71]. One exception within the network was SEPP1, which did not fall into any of the three biological groups but was functionally connected to GPX3. Both SEPP1 and GPX3 are known to be involved in selenium-dependent redox homeostasis[72] explaining the presence of SEPP1 in the clusterin interactome. The complete list of proteins in the network is provided in the Supporting Information (Table S4).

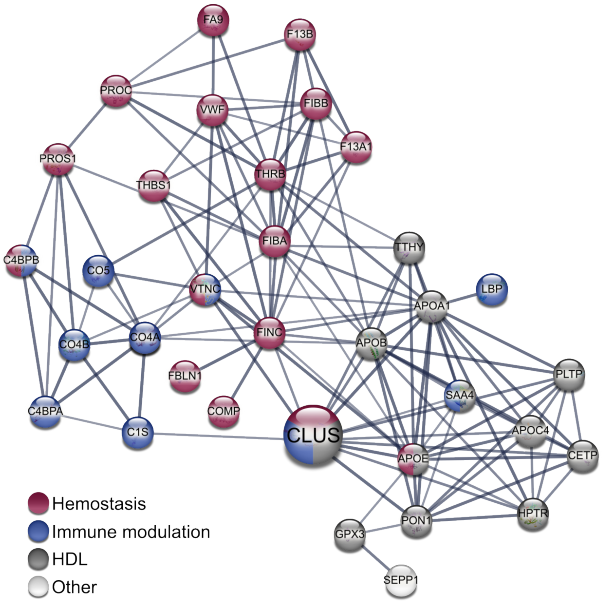

Figure 5: Clusterin interactome in human plasma, visualised using the STRING database. The network comprised 40 proteins significantly enriched following clusterin purification using Protocol 1. The network was mapped based on known and predicted protein–protein interactions. Edges represent confidence scores between proteins, with a minimum required interaction score of 0.700 (high confidence), and edge thickness corresponds to interaction strength. Protein nodes were grouped by biological function: immune modulation (blue), HDL particles (grey), and hemostasis pathway (red). SEPP1 (white) was the only protein in the network not assigned to the aforementioned groups.

Overall, the proposed AP-MS workflow revealed that the interactome of clusterin in human plasma is extensive. Notably, most proteins identified in the analysis have been previously reported to interact with clusterin, supporting the specificity of the observed interaction. This suggests that the detected proteins are likely to result from genuine interactions with clusterin or clusterin-containing complexes rather than from non-specific binding to the affinity resin, demonstrating that the proposed workflow is suitable for mapping the cluster interactome in complex biological matrices, such as human plasma.

Concluding Remarks

Clusterin is an extracellular chaperone implicated in a wide range of biological processes. Accordingly, analysis of clusterin in human biofluids provides valuable insight into its functional roles in pathological and physiological processes. However, the purification of clusterin from complex biological matrices remains challenging due to the high protein diversity, the presence of highly abundant species, and its inherent tendency to associate with multiple interaction partners. In this study, we established a versatile workflow combining affinity purification with bottom-up proteomics analysis to enable both targeted purification of clusterin and characterisation of its interactome in human plasma. This approach improves access to clusterin in complex biofluids and enables comprehensive analysis of clusterin-associated proteins in human plasma, providing complementary structural and functional insight into clusterin. Such capabilities are particularly relevant given clusterin's established role in neurodegenerative diseases, such as Alzheimer's and Parkinson's, where it modulates the aggregation of amyloid-β and α-synuclein[16]. Overall, this work establishes the foundation for future studies that aim to link clusterin-mediated protein interactions to biological functions and disease-related mechanisms.

DATA AVAILABILITY

The mass spectrometry proteomics data and the FragPipe workflow used have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD075110.

ASSOCIATED CONTENT

Supporting Information.

The supporting information is available free of charge.

Detailed experimental procedures, including purification workflows and buffer compositions (Table S1); mass spectrometry method parameters (Table S2); protein abundance plots comparing post sample loading incubation times (Figure S1); protein abundance plots following optimisation of washing and elution buffer compositions (Figure S2); STRING network analysis parameters (Table S3); and a complete list of proteins identified in the clusterin interactome (Table S4) (PDF). All combined_protein.tsv output files generated by FragPipe for each experimental condition tested (XLSX).

AUTHOR INFORMATION

Corresponding Author

Melissa Baerenfaenger − Division of Bioanalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, Amsterdam Institute of Molecular and Life Sciences, Vrije Universiteit Amsterdam, 1081 HV Amsterdam, The Netherlands; Centre for Analytical Sciences Amsterdam (CASA), The Netherlands; orcid.org/0000-0002-2855-924X; Email: j.m.baerenfaenger@vu.nl

Authors

Amalia Kontochristou − Division of Bioanalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, Amsterdam Institute of Molecular and Life Sciences, Vrije Universiteit Amsterdam, 1081 HV Amsterdam, The Netherlands; Centre for Analytical Sciences Amsterdam (CASA), The Netherlands; orcid.org/0009-0006-5988-0024

Nika Šimičić − Division of Bioanalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, Amsterdam Institute of Molecular and Life Sciences, Vrije Universiteit Amsterdam, 1081 HV Amsterdam, The Netherlands; Centre for Analytical Sciences Amsterdam (CASA), The Netherlands; Center for Proteomics and Metabolomics,

Leiden University Medical Center, Leiden 2333, ZA, The Netherlands; orcid.org/0009-0000-4381-6219

Anouk M. Rijs − Division of Bioanalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, Amsterdam Institute of Molecular and Life Sciences, Vrije Universiteit Amsterdam, 1081 HV Amsterdam, The Netherlands; Centre for Analytical Sciences Amsterdam (CASA), The Netherlands; orcid.org/0000-0002-7446-9907

Author Contributions

The manuscript was written with contributions from all authors. All authors have approved the final version. A. K. contributed to the experimental work, methodology development, conceptualisation, data acquisition, and wrote the original manuscript. N. Š. contributed to methodology development. A. M. R. contributed to supervision, funding acquisition, manuscript review, and editing. M. B. contributed to experimental work, methodology development, supervision, conceptualisation, funding acquisition, manuscript review, and editing.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AP-MS, affinity purification–mass spectrometry; CLUS, clusterin; DDA-PASEF, data-dependent parallel accumulation–serial fragmentation; DPBS, Dulbecco's phosphate-buffered saline; ESI, electrospray ionisation; FA, formic acid; FC, fold change; HDL, high-density lipoprotein; HPLC, high-performance liquid chromatography; LFQ-MBR, label-free quantification–Match Between Runs; MAC, membrane attack complex; SPE, solid-phase extraction; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; timsTOF, trapped ion mobility time-of-flight.

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